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Cristina Rafaela dos Santos Albuquerque Guimarães

Towards antioxidant and antitumor properties of wild medicinal plants traditionally used in Portugal: extracts, isolated flavonoids, and their human metabolites obtained by chemical synthesis

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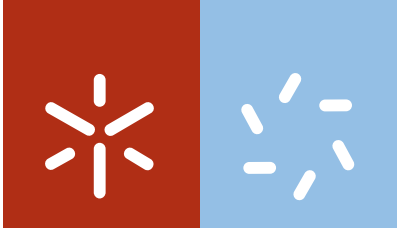

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Towards antioxidant and antitumor properties of wild medicinal plants traditionally used in Portugal: extracts, isolated flavonoids, and their human metabolites obtained by chemical synthesis

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Doutora Ana Maria Carvalho

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STATEMENT OF INTEGRITY

I hereby declare having conducted my thesis with integrity. I confirm that I have not used plagiarism or any form of falsification of results in the process of the thesis elaboration. I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho

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ABSTRACT

According their empirical relevance in traditional medicine and diets, six wild species from the Portuguese medicinal flora were selected as important sources of phenolic compounds, namely flavonoids. The present study aimed to characterize the phenolic composition and evaluate bioactive properties (antioxidant, antitumor and antiangiogenic) of methanolic extracts, infusions and decoctions prepared from *Chamaemelum nobile* and *Matricaria recutita*, and *Prunus spinosa*, *Arbutus unedo*, *Rosa micrantha* and *Rosa canina* fruits; and to synthesize derivatives/metabolites of some flavonoids present in the studied samples. The main phenolic compounds in *C. nobile* extract, decoction and infusion were flavonols, flavones, phenolic acids and derivatives. The extract gave the highest antioxidant activity in the β-carotene bleaching and TBARS formation inhibition assays. The highest radical scavenging activity and reducing power were observed in the infusion. The extract showed the highest antitumor (in different human tumor cell lines) and antiangiogenic (phosphorylation inhibition of VEGFR-2) activities. *M. recutita* infusion and decoction showed better results than the extract in the radical scavenging activity and β-carotene bleaching inhibition assays. The antitumor activity of the extract and infusion showed to be selective for HCT-15 and HeLa cell lines. Luteolin-*O*-acylhexoside was the most abundant flavonoid in the three preparations. 3-*O*-Caffeoylquinic acid was the most abundant phenolic compound in *P. spinosa*, quercetin 3-*O*-glucoside in *A. unedo*, and taxifolin in *R. micrantha* and *R. canina*. *P. spinosa* revealed to be the most rich in anthocyanins. Two different enriched phenolic extracts were prepared, in order to compare their bioactivity: non-anthocyanin phenolic compounds enriched extract (PE) and anthocyanins enriched extract (AE). *A. unedo* (PE) and *R. canina* (AE) presented the highest antioxidant activity in all the assays, and the highest antitumor activity was observed in *A. unedo* (PE and AE). The synthesis of derivatives/metabolites of flavonoids was performed using quercetin or quercetin 3-*O*-rutinoside (rutin) (identified in the studied samples) affording *O*-tri and different *O*-tetra protected (benzyl or methyl) quercetins that were submitted to glucuronidation attempts using acetobromo-α-D-glucuronic acid methyl ester in order to obtain possible precursors of human metabolites. As complex mixtures were obtained, further studies are needed to allow the formation of higher amounts of the required glucuronide derivatives to enable their isolation in a pure form.

RESUMO

De acordo com a sua relevância empírica na medicina tradicional e utilização em dietas, foram selecionadas seis espécies silvestres da flora medicinal Portuguesa, como fontes importantes de compostos fenólicos, nomeadamente flavonoides. O presente estudo teve como objetivo caracterizar a composição fenólica e avaliar as propriedades bioativas (antioxidante, antitumoral e antiangiogénica) de extratos, infusões e decocções preparadas a partir de *Chamaemelum nobile* e *Matricaria recutita*, e frutos de *Prunus spinosa*, *Arbutus unedo*, *Rosa micrantha* e *Rosa canina*; e sintetizar derivados/ metabolitos de alguns flavonoides presentes nas espécies estudadas. Os compostos fenólicos mais abundantes no extrato, decocção e infusão de *C. nobile* foram flavonóis, flavonas, ácidos fenólicos e derivados. O extrato mostrou a maior atividade antioxidante nos ensaios de inibição da descoloração do β-caroteno e da formação de TBARS. A maior atividade captadora de radicais livres e o maior poder redutor foram observados na infusão. O extrato mostrou maior atividade antitumoral (nas diferentes linhas celulares tumorais) e antiangiogénica (inibição da fosforilação do VEGFR-2). A infusão e a decocção de *M. recutita* mostraram maior atividade captadora de radicais livres e inibição da descoloração do β-caroteno do que o extrato. Os efeitos antitumorais do extrato e da infusão mostraram ser seletivos para as linhas celulares HeLa e HCT-15. A luteolina-*O*-acil-hexósido foi o flavonoide mais abundante nas três preparações. O ácido 3-*O*-cafeoilquínico foi o composto fenólico mais abundante em *P. spinosa*, enquanto que a quercetina 3-*O*-glucósido foi o maioritário em *A. unedo*, e a taxifolina foi o mais abundante em *R. canina* e *R. micrantha*. *P. spinosa* revelou o maior teor em antocianinas. Prepararam-se dois extratos fenólicos diferentes de forma a comparar a sua bioatividade: um extrato enriquecido em compostos fenólicos sem antocianinas (PE) e um extrato enriquecido em antocianinas (AE). *A. unedo* (PE) e *R. canina* (AE) apresentaram a maior atividade antioxidante em todos os ensaios, e a maior atividade antitumoral foi observada em *A. unedo* (PE e AE). A síntese de derivados/metabolitos dos flavonoides foram realizados usando a quercetina e rutina (quercetina 3-*O*-rutinósido) (identificados nas amostras estudadas) obtendo-se *O*-tri e diferentes *O*-tetra (benzil ou metil) quercetinas protegidas, que foram submetidas a tentativas de glucuronidação usando o éster metílico do ácido acetobromo-α-D-glucurónico a fim de obter possíveis precursores de metabolitos humanos. Tendo-se obtido misturas complexas, são necessários mais estudos para permitir a formação de quantidades mais elevadas dos glucuronidos requeridos, que possibilitem o isolamento dos compostos numa forma pura.

SCIENTIFIC PUBLICATIONS WITHIN THIS PhD THESIS

Articles in International Peer-Reviwed Journals:

[1] Rafaela Guimarães, Lillian Barros, Montserrat Dueñas, Ricardo C. Calhelha,Ana Maria Carvalho, Celestino Santos-Buelga, Maria João R.P. Queiroz, Isabel C.F.R. Ferreira. (2013). "Nutrients, phytochemicals and bioactivity of wild Roman chamomile: a comparison between the herb and its preparations", Food Chemistry, 136, 718-725.

[2] Rafaela Guimarães, Lillian Barros, Montserrat Dueñas, Ricardo C. Calhelha,Ana Maria Carvalho, Celestino Santos-Buelga, Maria João R.P. Queiroz, Isabel C.F.R. Ferreira. (2013). "Infusion and decoction of wild German chamomile: bioactivity and characterization of organic acids and phenolic compounds", Food Chemistry, 136, 947-954.

[3] Rafaela Guimarães, Lillian Barros, Montserrat Dueñas, Ricardo C. Calhelha,Ana Maria Carvalho, Celestino Santos-Buelga, Maria João R.P. Queiroz, Isabel C.F.R. Ferreira. (2013). "Characterization of phenolic compounds in wild fruits of Northeastern Portugal", Food Chemistry, 141, 3721-3730.

[4] Rafaela Guimarães, Lillian Barros, Ricardo C. Calhelha, Ana Maria Carvalho, Maria João R.P. Queiroz, Isabel C.F.R. Ferreira. (2014) "Bioactivity of different enriched phenolic extracts of wild fruits from Northeastern Portugal: A comparative study" Plant Foods Humam Nutrition 2014, 69, 37-42.

[5] Rafaela Guimarães, Ricardo C. Calhelha, Hugo J.C. Froufe, Rui M.V. Abreu, Ana Maria Carvalho, Maria João R.P. Queiroz, Isabel C.F.R. Ferreira. (2015). "Wild Roman chamomile extracts and phenolic compounds: enzymatic assays and molecular modelling studies with VEGFR-2 tyrosine kinase" Food and Function, Accepted 21st September 2015, DOI: 10.1039/c5fo00586h.

Proceedings in Scintific meetings:

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[2] Rafaela Guimarães, Lillian Barros, Filipa Reis, Montserrat Dueñas, Ana Maria Carvalho, Celestino Santos-Buelga, Maria João R.P. Queiroz, Isabel C.F.R. Ferreira. Phenolic profile of wild fruits of Rosa canina sl. from Northeast Portugal. The Second North and East European Congress on Food. NEEFood – 2013. 26-29 May 2013, Kyiv, Ukraine; 196p.

[3] Rafaela Guimarães, Lillian Barros, Ana Maria Carvalho, Maria João R.P. Queiroz, Isabel C.F.R. Ferreira. Composição química e propriedades antioxidantes de duas espécies silvestres de camomila do Nordeste de Portugal: camomila-alemã e camomila-romana. 16 a 19 de Setembro de 2012, Bragança, Portugal. ISBN:978-972-745-141-8.

[4] Rafaela Guimarães, Lillian Barros, Montserrat Dueñas, Ana Maria Carvalho, Celestino Santos-Buelga, Maria João R.P. Queiroz, Isabel C.F.R. Ferreira. Infusão e decocção da camomila romana silvestre são fontes de antioxidantes: caracterização do seu perfil fenólico. 16 a 19 de Setembro de 2012, Bragança, Portugal. ISBN:978-972-745-141-8.

Oral communications in scientific meetings:

[1] Rafaela Guimarães, Lillian Barros, Montserrat Dueñas, Ana Maria Carvalho, Maria João R.P. Queiroz, Celestino Santos-Buelga, Isabel C.F.R. Ferreira. Phenolic profile of *Arbutus unedo* L. and *Prunus spinosa* L. wild fruits. International Symposium on Profiling. 2nd - 4th September 2013, Caparica – Portugal. S17, 45p.

[2] Rafaela Guimarães, Lillian Barros, Montserrat Dueñas, Ana Maria Carvalho, Maria João R.P. Queiroz, Celestino Santos-Buelga, Isabel C.F.R. Ferreira. Phenolic profile of wild fruits of Rosa micrantha ex Sm. and Rosa canina L. from Northeast Portugal. 8º Encontro Nacional de Cromatografia, 2 a 4 de Dezembro de 2013, Covilhã. CO.05; pp. 59-60.

Poster communications in scientific meetings:

[1] Rafaela Guimarães, Maria João Sousa, Isabel C.F.R. Ferreira. Diferentes contribuições de óleos essenciais e fenóis para a actividade antioxidante de três plantas aromáticas (cedro, esteva e eucalipto), 2º Congresso Iberoamericano de Fitoterapia, 8 a 10 de Outubro de 2009, Lisboa, Portugal. P30, 105 p., Revista de Fitoterapia, 9 (S1).

[2] Rafaela Guimarães, Lillian Barros, Ana Maria Carvalho, Isabel C.F.R. Ferreira. Rosa micrantha as a powerfull source of antioxidants, International Symposium on the Patophysiology of Reactive Oxygen and Nitrogen Species, 10 a 21 de Maio de 2010, Salamanca, Espanha. PS5-11, 228p.

[3] Rafaela Guimarães, Lillian Barros, Montserrat Dueñas, Ana Maria Carvalho, Celestino Santos-Buelga, Maria João R.P. Queiroz, Isabel C.F.R. Ferreira. Comparative study of the phenolic profile and antioxidant properties of *Chamaemelum nobile*: infusion, decoction, and hydroalcoholic extract. XXVIth International Conference on Polyphenols, 23-26 June 2012, Florence, Italy. Polyphenol Communications 2012, volume II. T4-26; 483-484p.

[4] Rafaela Guimarães, Lillian Barros, Filipa Reis, Montserrat Dueñas, Ana Maria Carvalho, Celestino Santos-Buelga, Maria João R.P. Queiroz, Isabel C.F.R. Ferreira. Phenolic profile of wild fruits of Rosa canina sl. from Northeast Portugal. The Second North and East European Congress on Food. NEEFood – 2013. 26-29 May 2013, Kyiv, Ukraine; 196p.

[5] Rafaela Guimarães, Lillian Barros, Montserrat Dueñas, Ana Maria Carvalho, Maria João R.P. Queiroz, Celestino Santos-Buelga, Isabel C.F.R. Ferreira. Composition in anthocyanins of wild fruits from Northeastern Portugal. 7th International Workshop on Anthocyanins. 9th-1th September, 2013, Porto, Portugal. P46, 104p.

[6] Rafaela Guimarães, Lillian Barros, Ana Maria Carvalho, Maria João Sousa, Jorge Sá Morais, Isabel C.F.R. Ferreira. Chromatographic analysis of important phytochemicals in aromatic plants, 6º Encontro Nacional de Cromatografia, 14 a 16 de Dezembro 2009, Madeira; P.34, 147-148p.

[7] Rafaela Guimarães, João C.M. Barreira, Lillian Barros, Ana Maria Carvalho, Isabel C.F.R. Ferreira. Efeito do método de preparação e do tempo de armazenamento na actividade antioxidante de ervas aromáticas. XII Encontro Nacional da Sociedade Portuguesa de Química- 100 anos de Química em Portugal, 3 a 6 de Julho de 2011, Braga; RL-CP 2, 307p.

[8] Rafaela Guimarães, Lillian Barros, Ana Maria Carvalho, Maria João R.P. Queiroz, Isabel C.F.R. Ferreira. Composição química e propriedades antioxidantes de duas

espécies silvestres de camomila do Nordeste de Portugal: camomila-alemã e camomila-romana. 11º Encontro Nacional de Química dos Alimentos, 16 a 19 de Setembro de 2012, Bragança, Portugal, CP141, 191p.

[9] Rafaela Guimarães, Lillian Barros, Montserrat Dueñas, Ana Maria Carvalho, Celestino Santos-Buelga, Maria João R.P. Queiroz, Isabel C.F.R. Ferreira. Infusão e decocção da camomila romana silvestre são fontes de antioxidantes: caracterização do seu perfil fenólico. 11º Encontro Nacional de Química dos Alimentos, 16 a 19 de Setembro de 2012, Bragança, Portugal, CP170, 220p.

[10] Rafaela Guimarães, Lillian Barros, Montserrat Dueñas, Ricardo C. Calhelha, Ana Maria Carvalho, Celestino Santos-Buelga, Maria João R.P. Queiroz, Isabel C.F.R. Ferreira. Comparative study of the phenolic profile and antioxidant properties of *Chamaemelum nobile*: infusion, decoction, and hydroalcoholic extract. XXVIth International Conference on Polyphenols, 23 a 26 de Julho de 2012, Florence, Italy, volume 2, T426, 483-484.

[11] Rafaela Guimarães, Lillian Barros, Montserrat Dueñas, Ricardo C. Calhelha, Ana Maria Carvalho, Celestino Santos-Buelga, Maria João R.P. Queiroz, Isabel C.F.R. Ferreira. Phenolic compounds and bioactive properties of wild German and Roman chamomiles. 3º Encontro Nacional de Química Terapêutica e 1st Portuguese-Spanish-Brazilian Meeting on Medicinal Chemistry, 28 a 30 de Novembro de 2012, Aveiro, P103; 155p.

[12] Rafaela Guimarães, Lillian Barros, Ricardo C. Calhelha, Montserrat Dueñas, Ana Maria Carvalho, Maria João R.P. Queiroz, Celestino Santos- Buelga, Isabel C.F.R. Ferreira. Bioactivity and chemical characterization of phenolic and anthocyanin extracts of *Arbutus unedo* L. wild fruits. 1st Symposium on Medicinal Chemistry of University of Minho. 17 de Maio de 2013, Braga, Portugal; PC-39; 56p.

[13] Rafaela Guimarães, Lillian Barros, Ricardo C. Calhelha, Ana M. Carvalho, Maria João R.P. Queiroz, Isabel C.F.R. Ferreira. Phytochemicals and bioactivity in wild German and Roman chamomiles infusions. 1st Symposium on Medicinal Chemistry of University of Minho. 17 de Maio de 2013, Braga, Portugal; PC-40; 57p.

[14] Rafaela Guimarães, Ricardo C. Calhelha, Hugo J.C. Froufe, Rui M.V. Abreu, Ana M. Carvalho, Isabel C.F.R. Ferreira, Maria João R.P. Queiroz. Inhibition of the VEGFR-2 tyrosine kinase domain by witd Roman chamomile extracts and phenolic compounds. 2nd Symposium on Medicinal Chemistry of University of Minho, 8 de maio de 2015, Braga, Portugal. P33.

[15] Rafaela Guimarães, Lillian Barros, Ana Maria Carvalho, Maria João R.P. Queiroz, Isabel C.F.R. Ferreira. Composição química e propriedades antioxidantes de duas espécies silvestres de camomila do Nordeste de Portugal: camomila-alemã e camomila-romana. 16 a 19 de Setembro de 2012, Bragança, Portugal, CP141, 191p.

[16] Rafaela Guimarães, Lillian Barros, Montserrat Dueñas, Ana Maria Carvalho, Celestino Santos-Buelga, Maria João R.P. Queiroz, Isabel C.F.R. Ferreira. Infusão e decocção da camomila romana silvestre são fontes de antioxidantes: caracterização do seu perfil fenólico. 16 a 19 de Setembro de 2012, Bragança, Portugal, CP170, 220p.

[17] Rafaela Guimarães, Lillian Barros, Montserrat Dueñas, Ricardo C. Calhelha, Ana Maria Carvalho, Celestino Santos-Buelga, Maria João R.P. Queiroz, Isabel C.F.R Ferreira. Phenolic compounds and bioactive properties of wild German and roman chamomiles. 3º Encontro Nacional de Química Terapêutica, 28-30 Novembro 2012, Aveiro, Portugal, CP103, 155p.

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ABBREVIATTIONS LIST

AE	fruits anthocyanins enriched extract
ANOVA	one vary analysis of variance
AOAC	official methods of analysis
AP-1	transcription factor activator protein-1
ATP	adenosine triphosphate
BnBr	benzyl bromide
BRESA	Herbarium of the Escola Superior Agrária De Bragança
CBG	cytosolic β-glucusidase
CE	collision energy
CES	collision energy spread
CHS	enzyme chalcone synthase
CoA	Coenzyme A
COMT	catechol-*O*-methyltransferase
CYP	cytochrome
DAD/PDA	diode array detector
DEPT 135º	Distortionless enhancement by polarization transfer 135° angle
DMEM	Dulbecco's Modified Eagle Medium
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
DP	declustering potential
DPPH	2,2-diphenyl-1-picrylhydrazyl radical
DTT	dithiothreitol
dw	dry weight
EC_{50}	sample concentration providing 50% antioxidant activity or 0.5 absorvance in the reducing power assay
EDTA	ethylenediaminetetraacetic acid
EMS	enhanced mass spectrometer
EP	entrance potencial

EPI	enhanced product ion
ESI	electrospray ionization
FA	fatty acids
FAME	fatty acid methyl esters
FBS	fetal bovine serum
FRET based assays	enzymatic fluorescence ressonance energy transfer
fw	fresh weight
GC-FID	gas chromatography with flame ionization detection
GI_{50}	sample concentration that inhibits 50% of the cell growth
HBSS	Hanks' balanced salt solution
HCT-15	colon carcinoma cell
HeLa	Henrietta Lacks human cervical carcinoma
HepG2	hepatocellular carcinoma cell line
HMBC	Heteronuclear Multiple Bond Coherence
HPLC	high performance liquid chromatography
HSQC	Heteronuclear Single Quantum Correlation
IC50	concentration that provided 50% of VEGFR-2 inhibition.
IS	internal standard
LDL	Low Density Lipoprotein
LPH	Lactase phlorizin hydrolase
MCF-7	breast adenocarcinoma cell line
MD	molecular dinamics
MDA-TBA	malondialdehyde-thiobarbituric acid
MS	mass spectrometry
mu	atomic mass unit
MUFA	monounsaturated fatty acids
NCI-H460	human lung carcinoma cell line
NMR	Nuclear magnetic resonance
PDB	protein data bank
PDBQT	protein data bank file format

PE	fruits non-anthocyanin phenolic compounds enriched extract
PLP2	non-tumor primary culture of porcine liver cells
P-PST	phenol sulphotransferases
Psi	pound-force per square inch
PUFA	polyunsaturated fatty acids
RI	refraction index
RMSD	root mean square deviation
ROS	radical oxygen spicies
SD	standard desviation
SFA	saturated fatty acids
SGLT1	sodium-dependent glucose transporter 1
SRB	sulforhodamine B
ST	sulphotransferase
TBARS	thiobarbituric acid reactive substances
TCA	trichloroacetic acid
TFA	trifluoroacetic acid
UDP/UGT	glucuronosyl transferase
UFLC	ultra fast liquid chromatography
UV	ultraviolet radiation
VEGF	vascular endothelial growth factor
VEGFR-2	vascular endothelial growth factor receptor 2

Chapter 1.
Introduction

Chapter 1. Introduction

Ethnobotany is an interdisciplinary science which investigates the relationships between plants and human societies. The knowledge of plant use was widespread in ancient civilizations. The folk knowledge about medicinal use of plants has been transmitted for centuries primarily orally (Pieroni & Privitiera, 2014). The traditional use of medicinal plants seems to have survived across generations relatively intact, some of these traditions have changed or disappeared, and new interests in the use of medicinal and aromatic plants have emerged (Quave et al., 2012). Until the middle of the 19th century, plants were the main therapeutic agents used by humans, and even today their role in medicine is still relevant (Camejo-Rodrigues et al., 2003, Pieroni & Privitiera, 2014). Ethnopharmacology focuses on the understanding of local and indigenous use of the bioactive natural products (Staub et al., 2015). In recent years, much of the ethnopharmaceutical research has been focused to more specific approaches in order to evaluate and better understanding the biological and pharmacological effects of the medicinal plants (Heinrich, 2003) Phytochemical studies on medicinal plants are relatively abundant in the last years (Delgado, 1992). Studies that combine a phytochemical and a detailed pharmacological approach, or studies that explored their effects on health has increased. Considering the increasing interest in the chemical nature and the mechanisms of action of antioxidants from commonly used medicinal plants, the species selected in this work are certainly important sources of flavonoids. To study pharmacological effects that confirm the relevance of empirical uses of some wild species and their contribution to a good health condition is important. The plants involved in this work, were chosen according to data provided by ethnobotanical surveys. It is based on consented interviews carried out in Trás-os-Montes, Portugal, and these plants are some of the most mentioned and used in this region. This work aims at providing knowledge of the mechanisms involved in health-promoting properties of such species through their flavonoids and/or metabolites.

1.1 *Flowers heads of Chamaemelum nobile* and *Matricaria recutita*

1.1.1 Botanical characterization

Roman chamomile, *Chamaemelum nobile* L. is a perennial herb found in wild and cultivated habitats in western Europe, North America and Northern Africa. In traditional

medicine, chamomile flowers are used as an anti-inflammatory and spasmolytic beverage for stomach disorders (Melegari et al., 1988; Rossi et al., 1988). German chamomile, *Matricaria recutita* L., is an annual herbaceous flowering plant native to Europe. Both from the botanical family Asteraceae, these herbs have been used as herbal remedies for thousands of years (Crevin, 1990).

German chamomile (also known as *Matricaria recutita* L., *Chamomilla recutita* [L.] Rauschert, *Matricaria chamomilla* L., *Matricaria suaveolens* L.), is one of the most popular and widely used medicinal plants in the world. The plant has a long history of application in herbal medicine, which dates back to ancient Greece and Rome where it was referenced by Hippocrates, Galen and Asclepius (Tolouee et al., 2010).

1.1.2 Bioactive properties

Different classes of bioactive constituents are present in Roman chamomile (*C. nobile*), including phenolic compounds (Carnat et al., 2004; Tschan et al., 1996). Otherwise, *C. nobile* is tradicionally considered to be an antiseptic, antibiotic, disinfectant, bactericidal, fungicidal and vermifuge. It has been used for centuries as anti-inflammatory, antioxidant, mild astringent, mild sedative, antispasmodic, antibacterial and healing medicine (Ma et al., 2007). Oral dosage forms (decoctions and infusions) are used for the symptomatic treatment of gastrointestinal disorders and of the painful component of functional digestive symptoms. External applications of extracts and lotions are recommended as repellent and emollient, in the treatment of skin disorders and for eye irritation or discomfort of various etiologies. Furthermore, it is used as an analgesic in diseases of the oral cavity, oropharynx or both and as a mouthwash for oral hygiene (Srivastava et al., 2010). In this work some studies of Roman chamomile (*C. nobile*) will be presented, focusing in nutritional composition, phytochemicals whitin the herb and its infusion and decoction, and, antioxidant and antitumor activity (SubChapter 3.1) (Guimarães et al., 2013).

German chamomile (*M. recutita*) contains several classes of biologically active compounds including essential oils (Granzera et al., 2006; Petronilho et al., 2012) and several polyphenols (McKay & Blumberg, 2006; Nováková et al., 2010). It has also been used traditionally as a medicinal and pharmaceutical preparation, due to its anti-inflammatory, anti-spasmodic, analgesic, antibacterial, anti-allergic antioxidant and mild astringent properties, and healing characteristics (Maschi et al., 2008; McKay &

Blumberg, 2006; Weiss, 1988). Externally, German chamomile has been used to treat diaper rash, cracked nipples, chicken pox, ear and eye infections, disorders of the eyes including blocked tear ducts, conjunctivitis, nasal inflammation and poison ivy (Srivastava et al., 2010). The use of German chamomile beverages as medicinal preparations has a long tradition in various countries. Infusions and essential oils are used in a number of commercial products including soaps, detergents, perfumes, lotions, ointments, hair products, baked goods, confections, alcoholic beverages and herbal teas (Gupta et al., 2010). Traditionally, German chamomile flowers are prepared as an infusion with water, to make an herbal tea (Harbourne et al., 2009). Recent research supports this use and shows that these properties are partly due to the phenolic content (Maschi et al., 2008; McKay & Blumberg, 2006).

In fact, different studies dealing with *M. recutita* flowers are available in literature, including reports on antioxidant properties of its methanol extract (Barros et al., 2010; Miliauskas et al., 2004), antitumor potential of aqueous and organic extracts (Strivastava & Gupta, 2007; Strivastava & Gupta, 2009), and phenolic composition of methanolic extracts (Mulinacci et al., 2000; Nováková et al., 2010).

1.2 *Fruits of Arbutus unedo, Prunus spinosa, Rosa canina* and *Rosa micrantha*

1.2.1 Botanical characterization

The fleshy fruits of several shrubs and small trees are very popular and play a particular role in folk medicine and food systems in many regions of the world. *Arbutus unedo* L. (Goméz & Villar, 2004) from the Ericaceae, is a species widely distributed in the Mediterranean region and North Africa. Its fruits are a red aggregate drupe generally known as strawberry-tree berries. *Prunus spinosa* L. (Castroviejo et al., 2001), a deciduous shrub native to Europe from the Rosaceae family produces purple-blue drupes that have also been used as astringent, diuretic and purgative (Lust, 1980).

Rosa canina L. is a widespread shrub native to Europe and Western Asia. It is a common species of dog roses (Rosa sect. *Caninae*), a section of Rosa most widespread in Central Europe (Wissemann et al., 2006). For centuries rose hips of *Rosa canina* have been used for nutrition, medicinal purposes and also for their ornamental value. Hips are rarely eaten fresh; usually they are dried and/or processed and consumed in form of herbal tea, nectar, wine or marmalade (Uggla et al., 2005; Yildiz & Alpaslan, 2012). Native to Europe and Western Asia, in Portugal, *Rosa micrantha* Borrer ex Sm. is a dense deciduous shrub of

the edges of oak forests. *Rosa micrantha* is a species highly praised in the Northeastern Portuguese region due to its characteristic apple scent, even if only skillful users are able to distinguish *Rosa micrantha* from the other roses of the section. Medicinal and veterinary are the more mentioned uses of this particular species (Carvalho, 2010).

In several Portuguese regions, the fruits of *Arbutus unedo* and of Rosaceae species are commonly eaten raw, prepared in jams or macerated with sugar, honey and brandy to obtain a digestive and laxative liqueur, which is usually drunk after copious meals (Novais et al., 2004; Salgueiro, 2004; Camejo-Rodrigues, 2006; Carvalho, 2010).

1.2.2 Bioactive properties

Exotic or unusual foods, such as the fruits of *Arbutus unedo* (strawberry-tree), *Prunus spinosa* (blackthorn), *Rosa canina* (dog rose) and *Rosa micrantha* (wild rose) may have great potential for food industries as a source of colors and flavors, as well as bioactive molecules such as phenolic compounds for dietary supplements or functional foods (e.g. Barros et al., 2010; Guimarães et al., 2010; Barros et al., 2011; Barros et al., 2012; Barros et al., 2013; Morales et al., 2013).

Arbutus unedo L. use is very popular in Portugal, particularly made in a kind of strong brandy, the 'aguardente de medronho'. In several Portuguese regions (Trás-os-Montes, Alentejo and Algarve) the fruits are eaten raw or made in liqueurs, as well as, bark or roots decoctions, are used as anti-inflammatory, laxative, carminative, digestive, odontalgic and cardiotonic (Novais et al., 2004; Salgueiro, 2004; Camejo-Rodrigues, 2006; Carvalho, 2010). Some literature report the traditional use of the leaves as a diuretic, urinary antiseptic, antidiarrheal, astringent, depurative, against blenorrhagia, diabetes and as antihypertensive (Ziyyat et al., 1997). Experimental investigations have already shown that the aqueous extract of the plant exhibited antihypertensive (Ziyyat & Boussairi, 1998) and vasorelaxant (Ziyyat et al., 2002) activities.

There is great interested in the chemical characterization of the mentioned wild fruits traditionally consumed in Portugal by their medicinal and edible properties. Recent phytochemical studies performed revealed that they have antioxidant properties and important antioxidant molecules such as tocopherols and carotenoids (Barros et al., 2010; Guimarães et al., 2010).

Prunus spinosa fruits are commonly eaten raw, prepared in jams or macerated with sugar, honey and brandy to obtain a digestive and laxative liqueur, which is usually drunk after

copious meals; they have also been used as astringent, diuretic and purgative (Novais et al., 2004; Salgueiro, 2004; Camejo-Rodrigues, 2006; Carvalho, 2010). Extracts from the flowers have been used as diuretic, anti-inflammatory, spasmolytic and mild laxative and 'blood cleansing' agents (List & Hörhammer, 1971; Borkowski et al., 1994). In homeopathy products from fresh flowers are applied for circulatory system disorders (Wasilewski & Czelej, 1994; Olszewska & Wolbiś, 2001).

Moreover, rose hips, i.e. the pomaceous fruit of dog roses, *Rosa canina* L., have prophylactic and therapeutic activities against a wide range of ailments, including the inflammatory disorders arthritis (Rein et al., 2004; Kharazmi, 2008), rheumatism, gout, and sciatica, as antipyretic, for colds and infectious including influenza, against gastrointestinal disorders, to aid digestion, and to prevent gastric mucosa inflammation and gastric ulcer, for gallstones, biliary complaints, as a laxative, for disorders of the kidney and the lower urinary tract, as a diuretic, for dropsy and as an astringent (Orhan et al., 2007).

Rosa canina fruits are eaten raw as snacks and show prophylactic and therapeutic activities against a wide range of ailments, including the inflammatory disorders arthritis, rheumatism, gout, colds and gastrointestinal affections (Rein et al., 2004; Orhan et al., 2007; Carvalho, 2010). Beneficial health and cosmetic properties of *R. micrantha* fruits have been transmitted and tapped by rural people for centuries; they were used to prepare homemade remedies that prevented or healed several human disorders and animal diseases (Parada et al., 2009; Carvalho, 2010).

A few works on the phenolic composition of *A. unedo* fruits are available from Italy (Pawlowska et al., 2006), Portugal (Fortalezas et al., 2010; Tavares et al., 2010; Mendes et al., 2011) and Spain (Pallauf et al., 2008; Ganhão et al., 2010), *P. spinosa* from Spain (Ganhão et al., 2010) and *R. canina* from Norway (Hvattum, 2002), Poland (Fecka, 2009), Serbia (Tumbas et al., 2012) and Spain (Ganhão et al., 2010).

1.3 Phenolic compounds

1.3.1 Chemical features and biosyntesis

Flavonoids belong to a group of natural substances and comprised the three major classes of secondary compounds produced higher plants (Winkel-Shirley, 1996). They are universally present in fruits, vegetables, legumes, nuts, seeds, herbs, grains, bark, roots, steams, flowers, cereals, tea and wine (Middleton, 1998; Kanadaswami et al., 2005), and

constitute a relatively diverse family of aromatic molecules (Winkel-Shirley, 2001). More than 8.000 varieties of these compounds have been identified. The biosynthetic pathway leading to the major classes of flavonoid-derivatives are summarized in **Figure 1** (Dixon & Steele, 1999).

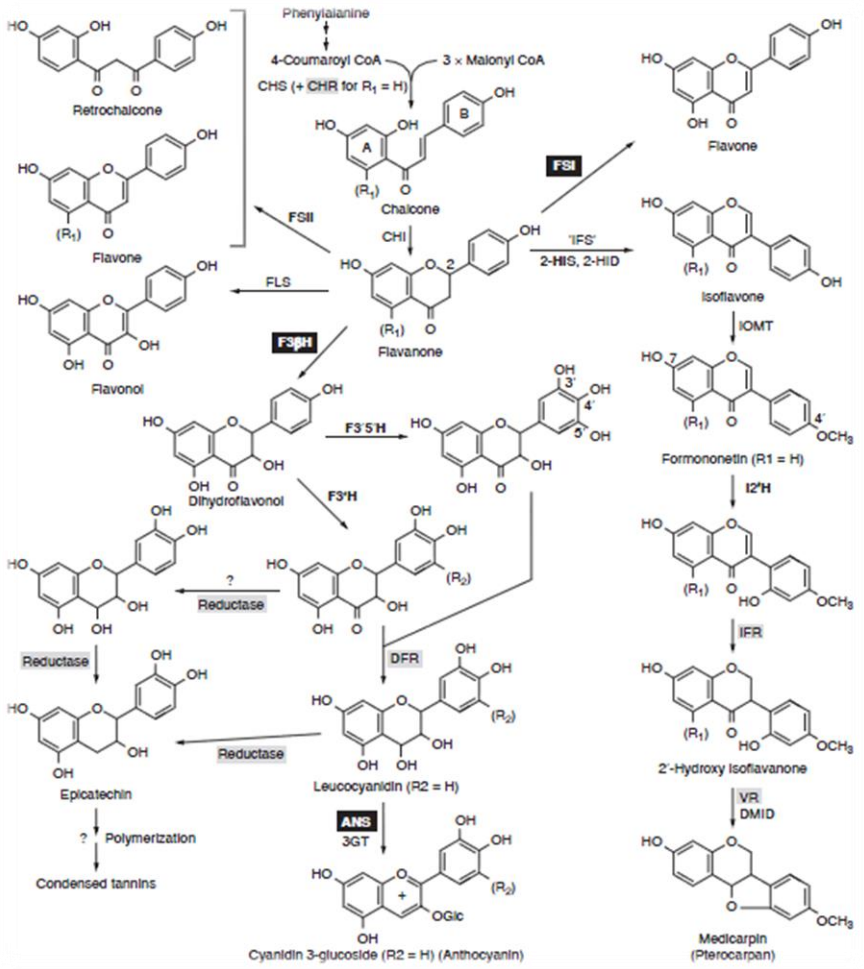


Figure 1. The biosynthesis of the major classes of flavonoid derivatives (Dixon & Steele, 1999).

All flavonoids are formed from a chalcone precursor, the product of the condensation of 4-coumaroyl CoA (a product of the central phenylpropanoid pathway) and three molecules of malonyl CoA (formed from acetate via a cytoplasmic form of acetyl CoA carboxylase) by the enzyme chalcone synthase (CHS) (Dixon & Steele, 1999). The flavonoids are phenyl substituted chromones (benzopyran derivatives) consisting of a 15-carbon basicskeleton (C_6-C_3-C_6) (Kanadaswami et al., 2005). The basic structural feature of flavonoid is a 2-phenyl-4*H*-chromene, consisting of two benzene rings (A and B) linked through a heterocyclic pyran ring (C) (Cushine & Lamb, 2005), the benzopyrane moiety being a 4*H*-chromene. Flavonoids occur as aglycones, glycosides and methylated

derivatives (Kumar & Pandey, 2013). The basic flavonoid structure is aglycone and as shown in **Figure 2**.

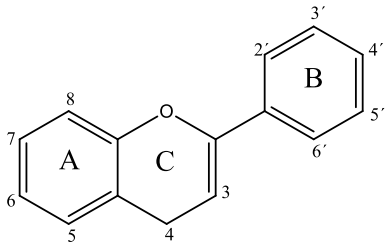


Figure 2. Basic structure of flavonoids: 2-phenyl-4*H*-chromenes (Cushine & Lambe, 2005).

Flavonoids are divided according to the chemical structure into several classes based on the degree of the oxidation of the C-ring, the hydroxylation pattern of the ring structure and the substitution of the 3-position (Spencer et al., 2009). Depending on their oxidation state and functional groups, flavonoids are further divided in six subclasses which general structures are shown in **Figure 3**: -**Flavones,** e.g. apigenin (5,7-dihydroxy and 4'-hydroxy) and luteolin (5,7-dihydroxy and 4'-hydroxy); -**Flavonols**, e.g. quercetin (5,7-dihydroxy and 3',4'-dihydroxy), myricetin (5,7-dihydroxy and 3',4',5'-trihydroxy) and kaempferol (5,7-dihydroxy and 4'-hydroxy); -**Flavanones,** e.g. naringenin (5,7-dihydroxy and 3',4'-dihydroxy) and hesperidin (5-hydroxy-7-*O*-diglycoside and 3'-hydroxy,4'-methoxy); -**Flavan-3-ols** or **catechins**, e.g. epicatechin (5,7-dihydroxy and 3',4'-dihydroxy) and gallocatechin (5,7-dihydroxy and 3',4',5'-trihydroxy); **Isoflavones,** e.g. genistein (5,7-dihydroxy and 4'-hydroxy) and **Anthocyanidins,** e.g. cyanidin (5,7-hydroxy and 3',4'-dihydroxy). The position of the aryl substituent divides the flavonoid class into flavonoids (at the 2-position) and isoflavonoids (at the 3-position). Flavonols differ from flavanones by the hydroxy group at the 3-position and the C2-C3 double bond (Narayana et al, 2001). Flavonoids are often hydroxylated in positions 3, 5, 7, 3', 4', and 5'. When glycosides are formed, the glycosidic linkage is normally located in positions 3 or 7 and the carbohydrates can be L-rhamnose, D-glucose, glucorhamnose, galactose, or arabinose (Middleton, 1984; Kumar & Pandey, 2013).

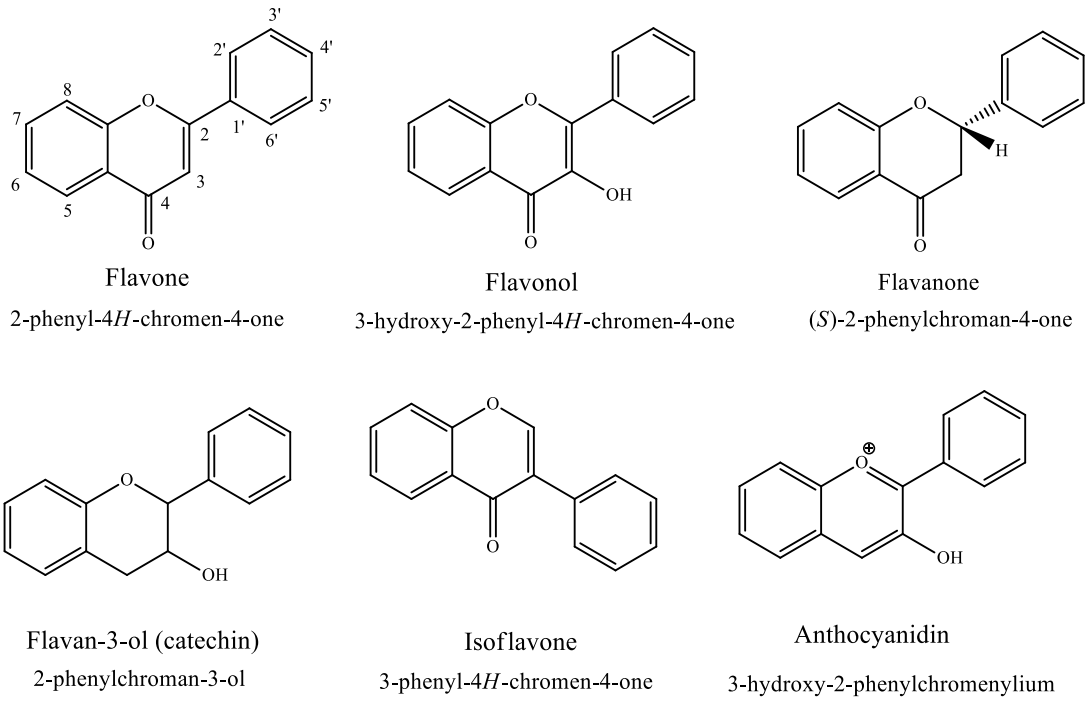


Figure 3. The basic groups of flavonoids (Mladěnka et al., 2010).

The flavones apigenin and luteolin are common in cereal grains and aromatic herbs (Pietta et al., 1995); flavonols quercetin and kaempferol are predominant in vegetables and fruits, and are constituents of the beverages green and black teas and red wine (Herrman, 1976). The flavanones are mainly found in citrus fruits, the flavan-3-ols (+)-catechin, (-)-epicatechin, (-)-epigallocatechin, and their gallate esters are widely present in tea leaves. Isoflavones are found most often in legumes, including soybeans, black beans, green beans, and chick peas. Anthocyanidins and their glycosides (anthocyanins) are natural pigments and are abundant in berries and red grapes (Hastam, 1989; Pietta, 2000).

The biochemical activities of flavonoids and their metabolites depend on their chemical structure and the relative position of various moieties on the molecule (Cody, 1988). The flavonoids can undergo modifications

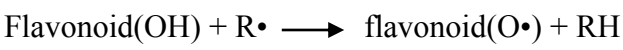
These molecules can suffer moditications on their aromatic cycles, including hydroxylations, methylations, or *O*-glycosylations of hydroxy groups as well as *C*-glycosylation directly to carbon atom of the flavonoid skeleton (Stobiecki & Kachlicki, 2006), hydrogenation, malonylation, sulphation acylations or prenylations, which account for the diversity within a compound class (Dixon & Pasinetti, 2010).

1.3.2 Bioactive properties

Flavonoids are plant phytochemicals that cannot be synthesized by humans (Peterson & Dwyer, 1998). Flavonoids have many biochemical, physiological and ecological functions in plants, and they are important in both plant productivity and animal nutrition (Forkmann & Martens, 2001). The function of flavonoids in flowers is to provide colours attractive to plant pollinators (Middleton & Chithan, 1993; Harborne & Williams, 2000). Flavonoids present in leaves promote physiological survival of plant by protecting it from the fungal infections and UV radiations. In addition, flavonoids are involved in photosensitization, respiration and photosynthesis control, morphogenesis, sex-determination and energy transfer (Cushine & Lamb, 2005). They can also function as stress protectants in plant cells by scavenging radical oxygen species (ROS) produced by the photosynthetic electron transport system (Harborne & Grayer, 1994). Flavonoids have antimicrobial properties and act as a deterrent for herbivores by limiting assimilation of dietary proteins and inhibiting digestive enzymes (Dixon et al., 2005; Marles et al., 2003; Scalbert, 1991). Flavonoids are present in daily human diet and have been reported to exert beneficial effects in disease prevention (Upadhyay & Dixit, 2015). Many studies have shown that these compounds play important biological, pharmacological, physiological, medicinal and epidemiological effects. The biological effects of dietary flavonoids include antiinflammatory, antiallergic, antimicrobial, hepatoprotective, antiviral, antibacterial, antithrombotic, cardioprotective, capillary strengthening, antidiabetic, anticarcinogenic, antineoplastic and vasodilatory actions (Middleton et al., 2000; Weber et al., 2003; Widlansky et al., 2005; Kanadaswami et al., 2005; Alvesalo et al., 2006).

Flavonoid metabolites may have a higher or lower biological activity than the parent drug, and result in a change of the overall cancer protective response (Nielsen et al., 2000). The flavonoids have applications as antibiotics, antidiarrheal, antiulcer and antitumorals. As anti-inflammatory agents, they have applications in the treatment of diseases such as hypertension, vascular fragility, allergies, hypercholesterolemia, and others (Bravo 1998; Yao et al., 2004; Ferguson et al., 2004; Ma et al., 2004; Moon et al., 2006). Epidemiologic studies suggest a protective role of dietary flavonoids with consumption of fresh fruits and vegetables against coronary heart disease (Pietta, 2000). There is increasing evidence for the health-protecting function of flavonoid compounds, such as antioxidative and antitumor activities (Forkmann & Martens, 2001). The best described property of almost

every group of flavonoids is their capacity to act as antioxidants (Kumar & Pandey, 2013). The protective effects of flavonoids in biological systems are ascribed to their capacity to transfer electrons free radicals, chelate metal catalysts (Ferrali et al., 1997), activate antioxidant enzymes (Elliott et al., 1992), reduce alpha-tocopherol radicals (Hirano et al, 2001) and inhibit oxidases (Cos et al., 1998). Flavonoids are oxidized by radicals (R•), resulting in a more stable, less-reactive radical; they stabilize the reactive oxygen species by reacting with the reactive compound of the radical. Because of the high reactivity of the hydroxy group of the flavonoids, radicals are made inactive, according to the following equation:



Selected flavonoids can directly scavenge superoxides, whereas other flavonoids can scavenge the highly reactive oxygen derived radical called peroxynitrite (Nijveld et al., 2001).The antioxidant activity of flavonoids and their metabolites *in vitro* depends upon the arrangement of functional groups about the nuclear structure (Heim et al., 2002). Several flavonoids have been reported to quench active oxygen species and inhibit *in vitro* oxidation of low-density lipoproteins and therefore reduce thrombotic tendency (Škerget et al., 2005). The antioxidant activity of flavonoids towards free radicals and reactive oxygen species, and their potential estrogenic and anticancer activity, such as antiproliferation, promotion of differentiation and apoptosis, draws attention to their health-protecting role in human diet and animal feed (Forkmann & Martens, 2001). *In vitro* data effectively and consistently demonstrate the antioxidant efficacy of structurally diverse flavonoids under many circumstances of oxidative stress (Heim et al., 2002). The chemopreventive properties of flavonoids are generally believed to reflect their ability to scavenge endogenous ROS (Galati & O'Brien, 2004). Interestingly, a recent review suggests that flavonoids may not act as conventional hydrogen-donating antioxidants but may exert modulatory actions in cells through actions at protein kinase and lipid kinase signaling pathways (Williams et al., 2004). Due to their capacity to inhibit LDL oxidation, flavonoids have demonstrated unique cardioprotective effects (Kondo et al., 1996; Mazur et al., 1999), from the ability to inhibit lipid peroxidation, chelate redox-active metals, and attenuate other processes involving ROS (Heim et al., 2002). Flavonoids are

extremely safe and associated with low toxicity, making them excellent candidates for chemopreventive agents (Moon et al., 2006). The cancer protective effects of flavonoids have been attributed to a wide variety of mechanisms, including free radical scavenging, modifying enzymes that activate or detoxify carcinogens, and inhibiting the induction of the transcription factor activator protein-1 (AP-1) activity by tumor promoters (Canivenc-Lavier et al., 1996; Shih et al., 2000). Isoflavonoids and flavonoids, have received particular attention as putative cancer protective agents in populations with low incidences of breast and prostate cancer (Adlercreutz et al., 1991). These compounds have been proposed as primary chemopreventive agents mainly due to their abilities to specifically inhibit tumor cell proliferation through blockade of signal transduction, their anti-oxidant effects and/or their anti-metastatic properties through an inhibition of tumor angiogenesis (Ramos, 2007; Pan et al. 2011; Zhao et al, 2009; He et al., 2009). The consummation of food products containing high amounts of flavonoids has been reported to lower the risk of various cancers and prevention of other diseases (Brusselmans et al., 2005; Arinç & Ylmaz, 2015).

1.3.3 Human metabolites

It is now established that polyphenols undergo substantial metabolism after being ingested by humans in dietary relevant amount and concentrations of plasma metabolites after a normal dietary intake rarely exceed nmol/L (Manach et al., 2005; Donovan et al., 2006). Polyphenols are extensively altered during first-pass metabolism so that, typically, the molecular forms reaching the peripheral circulation and tissues are different from those present in foods (Day et al., 2001; Day & Williamson, 2001; Graefe et al., 2001; Natsume et al., 2003; Setchell et al., 2003; Zhang et al., 2003). The absorption of dietary flavonoids liberated from the food by chewing will depend on its physicochemical properties such as molecular size, configuration, lipophilicity, solubility, and pKa. Absorption may also depend on dosage, vehicle of administration, antecedent diet, sex differences, and microbial population on the colon (Erlund et al., 2001; Kumar & Pandey, 2013). Importantly it is the chemical structure of polyphenols and not its concentration that determines the rate and extent of absorption and the nature of the metabolites circulating in the plasma (Pandey & Rizvi, 2009). The flavonoids can be absorved from small intestine or have go to the colon before absorption. It may depend upon structure of flavonoid, which is whether the glycoside or the aglycone (Erlund et al., 2001; Kumar

& Pandey, 2013). There are two major sites of flavonoid metabolism, the colon microflora, which (in addition to release aglycones) degrade flavonoids into phenolic acids, and in the liver (Rice-Evans, 2001) (**Table 1**).

Table 1.Enzymes involved in polyphenol metabolism.

Enzyme		Local	Function	Reference
CBG-Cytosolic β-glucusidase (EC2.2.1.1)		Especially found in liver	Catalyzes the hydrolysis of xenobiotic glycosides	Gopalan et al., 1992; Lamarco & Glew, 1986
LPH-Lactase phlorizin hydrolase (EC3.2.1.108)		Small intestine	Catalyzes the hydrolysis of a wide range of polyphenol glucoside	Day et al., 2000
COMT- Catechol-*O*-methyltransferase (EC2.1.1.6)		Variety of tissues	Methylates polyphenols	Nielsen et al., 1998
UDP- Glucuronosyl transferase (UDPGT/UGT/EC2.4.1.17)		Endoplasmatic reticulum	Catalyzes the conjugation of polyphenols to glucuronic acid	Yang et al., 1998; Mojarrabi & MacKenzie, 1998; Strassburg et al., 1998,1999.
	UGT1A	Intestine, liver, kidney		
	UGT1A1	Found in liver		
	UGT1A3			
	UGT1A4			
	UGT1A6			
	UGT1A9	Liver, kidney		
	UGT1A8	Liver, colon		
	UGT1A7	Human gastric epithelium		
	UGT1A10			
P-PST-Phenol sulphotransferases (SULT/EC 2.8.2.1)		Cytosolic enzymes		Coughtrie et al, 1998
	SULT1A1	Liver		
	SULT1A3	Colon		

The metabolism of flavonoids was initially thought to be mediated by cytochrome P450 enzymes (CYP P450 demethylates flavonols at the 4'-position and not at the 3'-position) (Nielsen et al., 1998; Walle, 2004).

For example, galangin (5,7-dihydroxyflavonol, **Figure 3**) is sequentially transformed to kaempferol (5,7,4'-trihydroxyflavonol, **Figure 3**) by the mechanism depending on cytochrome P450 reactions among which the CYP1A1 plays the major role (Silva et al., 1997; Silva et al., 1997). The metabolism of flavonoids starts in lumen of small intestine and post-absorption modifications also occur in the liver and other organs (Manach et al., 2005; Mullen et al., 2006). The degradation of a flavonoid skeleton occurs mainly in the gut. Bacterial ring scission of flavonoids results in degradation products, such as various phenolic acids. These metabolites can be again absorbed and consequently found in urine

(Hollman & Katan, 1997; Rice-Evans, 2001). They are absorbed through the gut barrier, or, for the non-absorbed fraction and the fraction re-excreted in the bile, by the colon microflora. All polyphenols are conjugated to form *O*-glucuronides, sulphate esters and *O*-methyl ethers (Scalbert, 2002). In 1995 Hollman et al., based on indirect evidence, proposed that flavonoid glycosides could be absorved intact in the small intestine, using the sodium-dependent glucose transporter 1 (SGLT1) (Hollman et al., 1995).

Most flavonoids, except for the subclass of catechins, are present in plants bound to sugars as β-glycosides. Because mammals lack the appropriate β-glycosidases, most of glycosides pass into the large intestine, where they are hydrolyzed by the microflora, in to their corresponding free aglycones (Griffiths & Barrow 1972; Bravo 1998). These are absorbed through the gut epithelium and methylated and/or conjugated with glucuronic acid or sulphate in the liver. This tissue has the capacity to oxidize (introduction of hydroxy groups), reduce (carbonyl groups), methylate (creation of *O*-methyl ethers) or conjugate (to form glucuronic- or sulphate-conjugates) flavonoids (Hackett, 1986).

Due to the conjugation reactions, no free flavonoid aglycones can be found in plasma or urine, except for catechins (Hollman, 2004). The flavonoids secreted with bile in intestine and those that cannot be absorbed by the small intestine are degraded in the colon by microflora, which also break down the flavonoid ring structure (**Figure 4**).

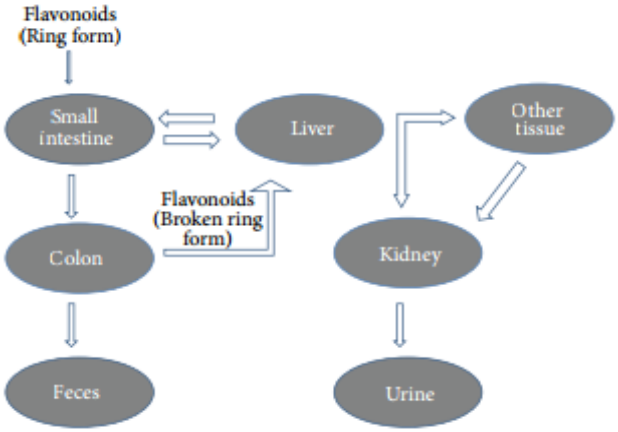


Figure 4. Compartments involved in the metabolism of flavonoids (Kumar & Pandey, 2013).

During absorption across the intestinal membrane, flavones, flavonols, isoflavones, and catechins are partly transformed in their glucuronides and sulphates (King et al., 1996).

Subsequently, this small fraction of the absorbed flavonoids is metabolized by the liver enzymes, resulting in more polar conjugates being excreted in the urine or returned to the duodenum. However, the major part of ingested flavonoids is not absorbed and is largely degraded by the intestinal microflora. The bacterial enzymes catalyze several reactions, producing several phenolic acids, depending on the structure of the flavonoid involved, as shown in **Figure 5** for rutin metabolism (Pietta, 2000).

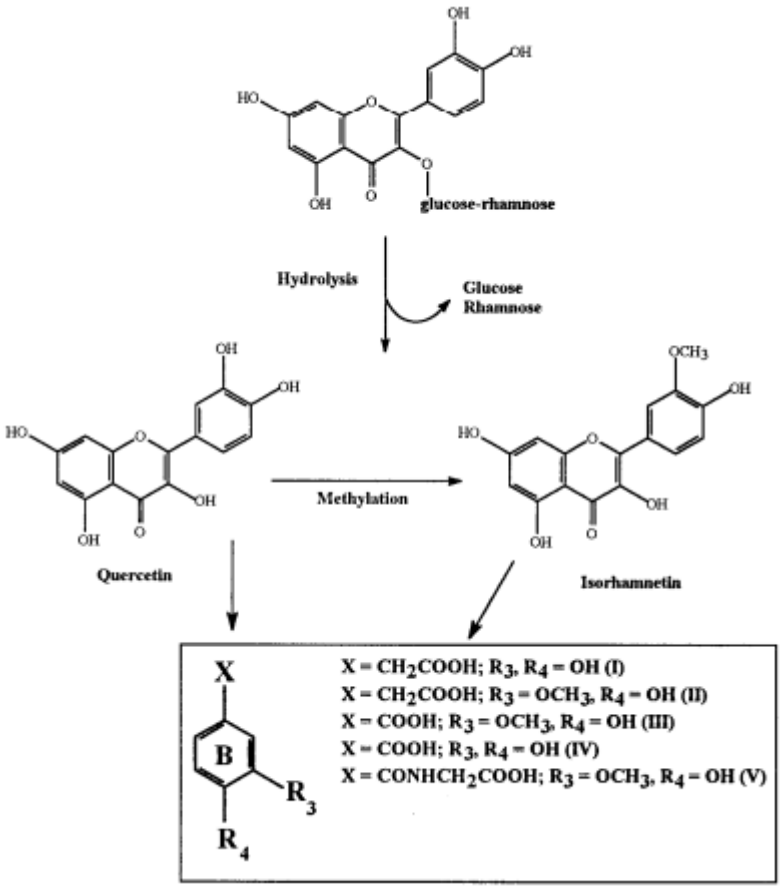


Figure 5. Metabolic conversion of rutin; (I) 3,4-dihydroxyphenylacetic acid, (II) 3-methoxy-4-hydroxyphenylacetic acid, (III) 3-methoxy-4-hydroxybenzoic acid, (IV) 3,4-dihydroxybenzoic acid and (V) 3-methoxy-4-hydroxyhippuric acid (Pietta, 2000).

These phenolic acids can be reabsorbed and subjected to conjugation and *O*-methylation in the liver and then may enter into the circulation (Pietta, 2000). The formation of anionic derivatives by conjugation with glucuronides and sulphate groups facilitates their urinary and biliary excretion and explains their rapid elimination. Another example of metabolic pathways for the conversion of dietary quercetin is shown in **Figure 6**.

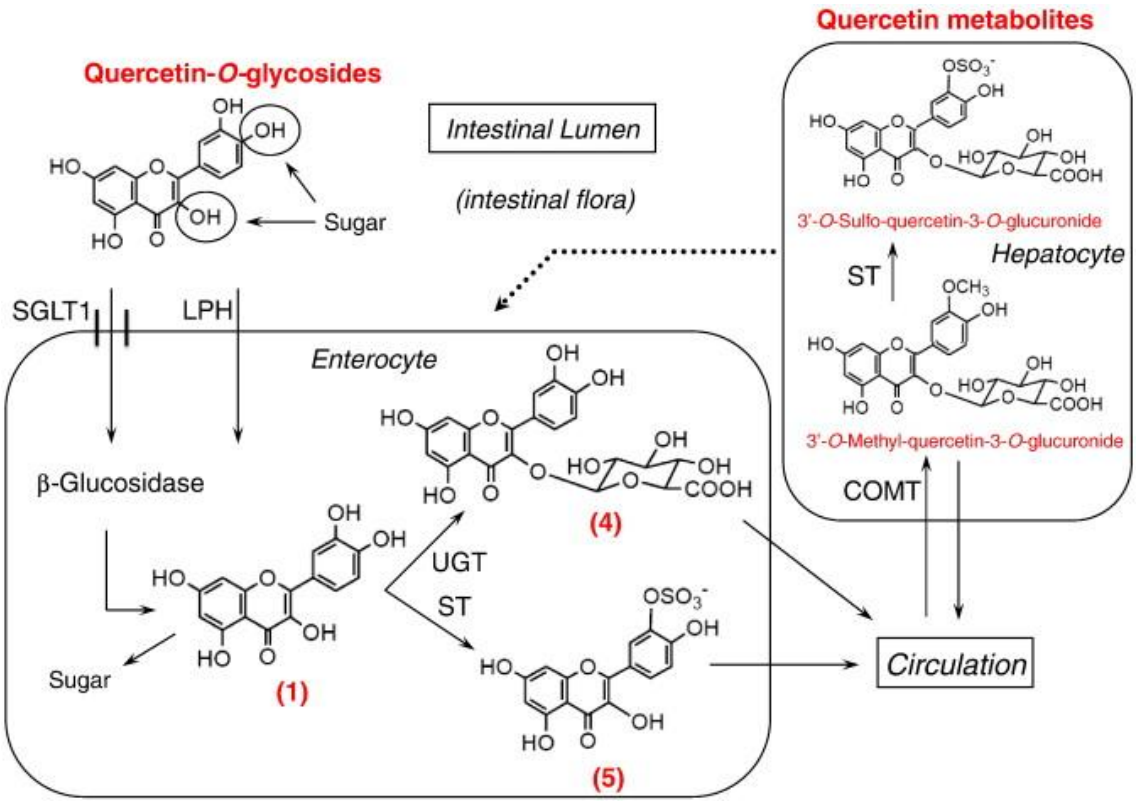


Figure 6. Major metabolic pathways for the conversion of dietary quercetin (Lotito et al., 2011).

Figure 6 showed the major metabolic pathways for the conversion of dietary quercetin. Quercetin, present in food as glycosides, is absorbed intact by SGLT1 and hydrolyzed by β-glucosidase in enterocytes. In addition, dietary glycosides of quercetin can be hydrolyzed before absorption by membrane-bound lactase phlorizin hydrolase (LPH). In the enterocyte, quercetin aglycone ("free" quercetin) (1) is mostly metabolized to quercetin-3-*O*-glucuronide (4) by UDP- glucuronosyl transferase (UGT) or to quercetin-3'-*O*-sulphate (5) by sulphate transferase (ST) and exported into the circulation. In the liver, the glucuronides undergo further metabolism, mainly by ST and catechol *O*-methyl transferase (COMT). These processes and enzymes combined are responsible for the metabolites detected in plasma and urine. In addition, quercetin may undergo enterohepatic circulation by its excretion through bile and subsequent intestinal reabsorption (Lotito et al., 2011).

The metabolism of most other dietary polyphenols in humans are comparable in several ways: (a) glycosides are generally not found in plasma or urine in the form ingested, (b)

the major forms in plasma and urine are sulphate and glucuronate conjugates of the parent aglycones, (c) methylation may occur on polyphenols that contain *ortho*-hydroxy functional groups, and (d) aglycones are absent or constitute only a very small proportion of the total amount of polyphenols present, except in green tea catechins, of which aglycones can constitute a significant proportion of the total amount in plasma (Hollman et al., 1997).

1.4 Objectives of the present study and general overview

The main objectives of our study were to:

1. Chemically characterize wild species selected according their empirical relevance in traditional medicine and diets such as *Chamaemelum nobile, Matricaria recutita, Prunus spinosa, Arbutus unedo, Rosa micrantha* and *Rosa canina.*
2. Evaluate the bioactive properties, including antioxidant, antitumor and phosphorylation inhibition of the vascular endothelium growth factor receptor-2 (VEGFR-2), of methanolic extracts, infusions and decoctions prepared from the mentioned species.
3. Synthesize derivatives/metabolites of some flavonoids present in the studied samples.

The nutritional characterization of *C. nobile* included the determination of macronutrients, free sugars, fatty acids, tocopherols and carotenoids. The herb and its decoction and infusion were submitted to a detailed analysis of phytochemicals (phenolic compounds and organic acids) and bioactivity evaluation. The antioxidant activity was determined by free radicals scavenging activity, reducing power and inhibition of lipid peroxidation; the antitumor potential was tested in human tumor cell lines (breast, lung, colon, cervical and hepatocellular carcinomas); and their toxicity was evaluated using a porcine liver primary cell culture established in our laboratory. Furthermore, the antiangiogenic activity (phosphorylation inhibition of VEGFR-2) of *C. nobile* extracts (methanolic extract and infusion) and their main phenolic compounds (apigenin, apigenin-7-*O*-glucoside, caffeic acid, chlorogenic acid, luteolin, luteolin-7-*O*-glucoside) was evaluated through enzymatic assays using the tyrosine kinase intracellular domain of VEGFR-2. To better understand the phosphorylation inhibition mechanism of the

tyrosine kinase receptor by luteolin, apigenin and apigenin-7-*O*-glucoside, docking studies were performed.

M. recutita infusion and decoction, the most consumed preparation of this herb, were characterized in terms of organic acids and phenolic composition, and evaluated for their bioactive properties (antioxidant and antitumor activities, and toxicity).

Finally, the *in vitro* antioxidant and antitumor properties of enriched phenolic extracts (non-anthocyanin phenolic compounds enriched extract and anthocyanins enriched extract) of *A. unedo*, *P. spinosa*, *R. micrantha* and *R. canina* wild fruits were evaluated and compared in order to clarify anthocyanins contribution for bioactivity and the advantageous of using purified/enriched instead of crude phenolic extracts.

As quercetin is present in all the wild species studied it was chosen to be used in the synthesis of some derivatives/metabolites resulting for the human diet. Benzylation and methylation reactions in order to protect some hydroxy groups were performed. Methylated compounds can also be metabolites. Benzylated and methylated compounds were submitted to glucuronidation reactions.

Chapter 2.

Chemical characterization and bioactive properties of the wild plants: Material and Methods

Chapter 2. Chemical characterization and bioactive properties of the wild plants: Material and Methods

2.1. Samples of plant materials

Plant materials used in this study were selected from culturally significant species previously inventoried in several ethnobotanical surveys conducted in Portugal (e.g. Novais et al., 2004; Salgueiro, 2004; Camejo-Rodrigues, 2006; Carvalho, 2010) and reported as widely used for homemade remedies in folk medicine, and for food purposes. Accordingly, it has been documented that some edible parts of these species (e.g. flowers and fruits) were claimed to have important medicinal properties and empirically considered as good sources of vitamins (Carvalho, 2010).

The selected species and the different types of plant materials collected are described in the following subsections. Gathering sites, plant parts or organs and the best period of the year to collect each species/material took into account local knowledge. Healers and experienced consumers' criteria were also considered, particularly those criteria concerning specific food processing or requirements for safe herbal dosages forms, such as infusion and decoction.

Voucher specimens of all studied species were deposited in the Herbarium of the Escola Superior Agrária de Bragança (BRESA).

2.1.1. Chamaemelum nobile

Chamaemelum nobile L. was gathered during the flowering season (June-July 2010) from wild populations located in grasslands in Bragança, Trás-os-Montes, Northeastern Portugal. Samples consisted of pieces of about 8 cm, corresponding to terminal soft leafy stems and inflorescences with flowers fully open and functional, picked up in plants randomly selected in a meadow of about a hectare. Morphological key characters from Rothmaler (2007) were used for plant identification. Plant material from different specimens was put together in a single final sample for analysis. The final sample was lyophilized (FreeZone 4.5, Labconco, Kansas, USA), reduced to a fine dried powder (20 mesh) and mixed to obtain homogenate sample.

2.1.2. Matricaria recutita

Matricaria recutita L. flower heads and leafy flowering stems of about 15 cm long were collected in 2009, in late spring and early summer, in the Natural Park of Montesinho territory, Trás-os-Montes, Northeastern Portugal. Plant material was collected from different specimens randomly chosen within a wild population. Morphological key characters from Rothmaler (2007) were used for plant identification. The sample was lyophilized (FreeZone 4.5, Labconco, Kansas, USA), reduced to a fine dried powder (20 mesh) and mixed to obtain homogenate sample.

2.1.3. Arbutus unedo, Prunus spinosa, Rosa canina and *Rosa micrantha*

The fruits of *Arbutus unedo* L. (strawberry-tree) from Ericaceae, and the Rosaceae species *Prunus spinosa* L. (blackthorn), *Rosa canina* sl. (dog rose) and *Rosa micrantha* Borrer ex Sm. (similar to eglantine rose) were also gathered in the Natural Park of Montesinho territory, in Trás-os-Montes, Northeastern Portugal. From each species, several individuals growing in a selected plot of aproximatelly one hectare were casually marked in order to collect the mature fruits. Strawberry-tree berries were picked fully ripened in November 2008; well matured sloes and dog rose hips were gathered in late September 2008. *R. micrantha* overripen hips, that is fleshy and soft dark red fruits, were collected in late autumn 2009. The ripeness degree of the fruits was established visually using the informant´s criteria of colour and texture, according to growth conditions of the season of the year. Considering that the state of ripeness was based in a subjective criterium, in order to obtain homogeneity within the samples of the same plant species, the colour of the removed ripened fruits from each plant was compared using the Royal Horticultural Society Colour Charts Edition V.

Morphological key characters from the Flora Iberica (Castroviejo et al., 2001; Goméz & Villar, 2004) were used for confirming plant identification. The fruits including the seeds were lyophilized (Ly-8-FM-ULE, Snijders, Holland) and stored in the deep-freezer at -20 ºC for subsequent analysis.

2.2. Standards and reagents

HPLC-grade acetonitrile was obtained from Merck KgaA (Darmstadt, Germany). Formic, acetic and trifluoroacetic acid (TFA) were purchased from Prolabo (VWR International,

France). Acetonitrile 99.9%, *n*-hexane 95% and ethyl acetate 99.8% were of HPLC grade from Fisher Scientific (Lisbon, Portugal). The fatty acids methyl ester (FAME) reference standard mixture 37 (standard 47885-U) was purchased from Sigma (St. Louis, MO, USA), as also other individual fatty acid isomers, sugars (D-(-)-Fructose, D-(+)-Glucose, D-(+)-Sucrose, D-(+)-Trehalose), tocopherols (α-, β-, γ-, and δ-isoforms) and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) standards. Racemic tocol, 50 mg/mL, was purchased from Matreya (PA, USA). The phenolic compound standards (apigenin-6-*C*-glucoside; apigenin-7-*O*-glucoside; caffeic acid; chlorogenic acid; ferulic acid; *p*-coumaric acid; isorhamnetin-3-*O*-glucoside; kaempferol-3-*O*-rutinoside; luteolin-6-*C*-glucoside; luteolin-7-*O*-glucoside; myricetin; myricetin-3-*O*-glucoside; protocatechuic acid; quercetin 3-*O*-glucoside and quercetin-3-*O*-rutinoside) were from Extrasynthese (Genay, France). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Fetal bovine serum (FBS), L-glutamine, Hank's balanced salt solution (HBSS), trypsin-EDTA (ethylenediaminetetraacetic acid), nonessential amino acids solution (2mM), penicillin/streptomycin solution (100 U/mL and 100 mg/mL, respectively), RPMI-1640 and DMEM media were from Hyclone (Logan, USA). Acetic acid, ellipticine, sulforhodamine B (SRB), trypan blue, trichloroacetic acid (TCA) and Tris and all organic acids standards (L-ascorbic acis; citric acid; fumaric acid; malic acid; shikimic acid; succinic acid; oxalic acid and quinic acid) were from Sigma Chemical Co. (Saint Louis, USA). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

2.3. Nutritional characterization

2.3.1. Crude composition

Chamaemelum nobile was analysed for chemical composition (moisture, proteins, fat, carbohydrates and ash) using the AOAC procedures (AOAC, 1995). The crude protein content (N × 6.25) was estimated by the macro-Kjeldahl method; the crude fat was determined by extracting a known weight of powdered sample with petroleum ether, using a Soxhlet apparatus; the ash content was determined by incineration at 600 ± 15 ºC. Total carbohydrates were calculated by difference. Energy was calculated according to the following equation: Energy (kcal) = 4 × (g protein + g carbohydrate) + 9 × (g fat).

2.3.2. Sugars composition

Free sugars of *Chamaemelum nobile* were determined by a High Performance Liquid Chromatography (HPLC) system consisted of an integrated system with a pump (Knauer, Smartline system 1000), degasser system (Smartline manager 5000) and auto-sampler (AS-2057 Jasco), coupled to a refraction index detector (RI detector Knauer Smartline 2300) as previously described by the authors (Pereira et al.,2012). Sugars identification was made by comparing the relative retention times of sample peaks with standards. Data were analyzed using Clarity 2.4 Software (DataApex). Quantification was based on the RI signal response of each standard, using the internal standard (IS, raffinose) method and by using calibration curves obtained from commercial standards of each compound. The results were expressed in g per 100 g of dry weight.

2.3.3. Fatty acids composition

Fatty acids of *Chamaemelum nobile* were determined after a transesterification procedure as described previously by the authors (Pereira et al., 2012), using a gas chromatographer (DANI 1000) equipped with a split/splitless injector and a flame ionization detector (GC-FID). Fatty acid identification was made by comparing the relative retention times of FAME peaks from samples with standards. The results were recorded and processed using CSW 1.7 software (DataApex 1.7). The results were expressed in relative percentage of each fatty acid.

2.3.4. Tocopherols composition

Chamaemelum nobile tocopherols were determined following a procedure previously optimized and described by the authors (Pereira et al., 2012). Analysis was performed by HPLC (equipment described above), and a fluorescence detector (FP-2020; Jasco) programmed for excitation at 290 nm and emission at 330 nm. The compounds were identified by chromatographic comparisons with authentic standards. Quantification was based on the fluorescence signal response of each standard, using the IS (tocol) method and by using calibration curves obtained from commercial standards of each compound. The results were expressed in mg per 100 g of dry weight.

2.3.5. Carotenoids composition

β-carotene and lycopene of *Chamaemelum nobile* were determined following a procedure previously described by Nagata & Yamashita (1992). A fine dried powder (500 mg) was vigorously shaken with 10 mL of acetone–hexane mixture (4:6) for 1 min and filtered through Whatman No. 4 filter paper. The absorbance of the filtrate was measured at 453, 505, 645 and 663 nm. Content of β-carotene and lycopene were calculated according to the following equations:

β-carotene (mg/100mL) = $0.216 \times A_{663} - 1.220 \times A_{645} - 0.304 \times A_{505} + 0.452 \times A_{453}$;

Lycopene (mg/100mL) = $-0.0458 \times A_{663} + 0.204 \times A_{645} - 0.304 \times A_{505} + 0.452 \times A_{453}$;

and further expressed in mg per 100 g of dry weight (dw).

2.4. Preparation of extracts

2.4.1. Methanolic extracts

2.4.1.1. *Chamaemelum nobile* and *Matricaria recutita*:

The methanolic extracts were prepared from the lyophilized plant material. Each sample (1 g) was extracted by stirring with 25 mL of methanol (25 ºC at 150 rpm) for 1 h and subsequently filtered through Whatman No. 4 paper. The residue was then extracted with 25 mL of methanol (25 ºC at 150 rpm) for 1 h. The combined methanolic extracts were evaporated at 40 ºC (rotary evaporator Büchi R-210) to dryness.

2.4.1.2. Arbutus unedo, Prunus spinosa, Rosa micrantha and *Rosa canina*

2.4.1.2.1. Non-anthocyanin phenolic compounds enriched extract (PE):

Each sample (1 g) was extracted with 30 mL of methanol:water 80:20 (*v/v*) at room temperature, 150 rpm, for 1 h. The extract was filtered through Whatman nº4 paper. The residue was then re-extracted twice with additional 30 mL portions of methanol:water 80:20 (*v/v*). The combined extracts were evaporated at 35 ºC (rotary evaporator Büchi R-210) to remove methanol. For purification, the extract solution was deposited onto a C-18 SepPak® Vac 3cc cartridge (Phenomenex), previously activated with methanol followed by water; sugars and more polar substances were removed by passing through 10mL of water and phenolic compounds were further eluted with 5mL of methanol. The methanolic extract obtained (designated by phenolic extract) was concentrated under vacuum and stored at 4 ºC for further use.

2.4.1.2.2. Anthocyanins enriched extract (AE):

Each sample (1 g) was extracted with 30 mL of methanol containing 0.5% TFA, and filtered through a Whatman nº4 paper. The residue was then re-extracted twice with additional 30 mL portions of 0.5% TFA in methanol. The combined extracts were evaporated at 35 ºC to remove the methanol, and re-dissolved in water. For purification, the extract solution was deposited onto a C-18 SepPak® Vac 3cc cartridge (Phenomenex), previously activated with methanol followed by water; sugars and more polar substances were removed by passing through 10 mL of water and anthocyanin pigments were further eluted with 5 mL of methanol:water (80:20, v/v) containing 0.1% TFA. The methanolic extract (designated by anthocyanins extract) was concentrated under vacuum, lyophilized and stored at 4 ºC for further use.

2.4.1.2.3. Phenolic acids, flavones/ols and flavan-3-ols*:*

Each sample (1 g) was extracted with 30 mL of methanol:water 80:20 (*v/v*) at room temperature, 150 rpm, for 1 h. The extract was filtered through Whatman nº4 paper. The residue was then re-extracted twice with additional 30 mL portions of methanol:water 80:20 (*v/v*). The combined extracts were evaporated at 35 ºC (rotary evaporator Büchi R-210) to remove methanol. For purification, the aqueous phase was deposited onto a C-18 SepPak® Vac 3cc cartridge (Phenomenex), previously activated with methanol followed

by water; sugars and more polar substances were removed by passing through 10mL of water and the purified extract was further eluted with 5 mL of methanol. The methanolic extract was concentrated under vacuum, and re-dissolved in 1mL of 20% aqueous methanol and filtered through a 0.22 μm disposable LC filter disk for HPLC analysis.

2.4.2. Aqueous extracts

2.4.2.1. Decoctions of *Chamaemelum nobile* and *Matricaria recutita*

Each sample (1 g) was added to 200 mL of distilled water, heated (heating plate, VELP scientific) and boiled for 5 min. The mixture was left to stand at room temperature for 5 min more, and then filtered under reduced pressure. The obtained decoctions were frozen and lyophilized.

2.4.2.2. Infusions of *Chamaemelum nobile* and *Matricaria. recutita*

Each sample (1 g) was added to 200 mL of boiling distilled water and left to stand at room temperature for 5 min, and then filtered under reduced pressure. The obtained infusions were frozen and lyophilized.

2.5. Analysis of phytochemicals in the extracts

2.5.1. Organic acids

Organic acids were determined in *Chamaemelum nobile* and *Matricaria recutita* following a procedure previously optimized and described by the authors (Barros,et al., 2012). Analysis was performed by ultra fast liquid chromatograph (UFLC) coupled to photodiode array detector (PDA), using a Shimadzu 20 A series UFLC (Shimadzu Corporation). Detection was carried out in a PDA, using 215 nm and 245 nm as preferred wavelengths. The organic acids were quantified by comparison of the area of their peaks recorded at 215 nm with calibration curves obtained from commercial standards of each compound. The results were expressed in g per 100 g of lyophilized methanolic extract/decoction/infusion.

2.5.2. Phenolic compounds

2.5.2.1. In *Chamaemelum nobile* and *Matricaria recutita*

Phenolic compounds were determined by HPLC (Hewlett-Packard 1100, Agilent Technologies, Santa Clara, USA) as previously described by the authors (Barros et al., 2012). Double online detection was carried out in the diode array detector (DAD) using 280 nm and 370 nm as preferred wavelengths and in a mass spectrometer (API 3200 Qtrap, Applied Biosystems, Darmstadt, Germany) connected to the HPLC system via the DAD cell outlet. The phenolic compounds were characterized according to their UV and mass spectra and retention times, and comparison with authentic standards when available. For quantitative analysis, calibration curves were prepared from different standard compounds. The results were expressed in g per 100 g of lyophilized methanolic extract/decoction/infusion.

2.5.2.2. In *Arbutus unedo, Prunus spinosa, Rosa micrantha* and *Rosa canina*

2.5.2.2.1. Phenolic acids and flavones/ols

The extracts were analysed using a Hewlett-Packard 1100 chromatograph (Agilent Technologies) with a quaternary pump and a diode array detector (DAD) coupled to an HP Chem Station (rev. A.05.04) data-processing station. A Waters Spherisorb S3 ODS-2 C_{18}, 3 μm (4.6 mm × 150 mm) column thermostatted at 35 °C was used. The solvents used were: (A) 0.1% formic acid in water, (B) acetonitrile. The elution gradient established was 10% B to 15% B over 5min, 15–25% B over 5 min, 25–35% B over 10 min, isocratic 50% B for 10 min, and re-equilibration of the column, using a flow rate of 0.5 mL/min. Double online detection was carried out in the DAD using 280 nm and 370 nm as preferred wavelengths and in a mass spectrometer (MS) connected to HPLC system via the DAD cell outlet. MS detection was performed in an API 3200 Qtrap (Applied Biosystems, Darmstadt, Germany) equipped with an ESI source and a triple quadrupole-ion trap mass analyzer that was controlled by the Analyst 5.1 software. Zero grade air served as the nebulizer gas (30 psi) and turbo gas for solvent drying (400 ºC, 40 psi). Nitrogen served as the curtain (20 psi) and collision gas (medium). The quadrupols were set at unit resolution. The ion spray voltage was set at -4500 V in the negative mode. The MS detector was programmed for recording in two consecutive

modes: Enhanced MS (EMS) and enhanced product ion (EPI) analysis. EMS was employed to show full scan spectra, so as to obtain an overview of all of the ions in sample. Settings used were: declustering potential (DP) -450 V, entrance potential (EP) -6 V, collision energy (CE) -10 V. EPI mode was performed in order to obtain the fragmentation pattern of the parent ion(s) in the previous scan using the following parameters: DP -50 V, EP -6 V, CE -25 V, and collision energy spread (CES) 0 V. Spectra were recorded in negative ion mode between *m*/*z* 100 and 1000. The phenolic compounds present in the samples were characterised according to their UV and mass spectra and retention times compared with standards when available. For the quantitative analysis of phenolic compounds, a 5-level calibration curve was obtained by injection of known concentrations (2.5-100 μg/mL) of different standards compounds: caffeic acid ($y = 617.91x – 691.51$; $R^2 = 0.9991$); chlorogenic acid ($y = 600.27x –763.62$; $R^2 = 0.9998$); *p*-coumaric acid ($y = 884.6x + 184.49$; $R^2 = 0.9999$); gallic acid ($y = 556.94x – 738.37$; $R^2 = 0.9988$); isorhamnetin 3-*O*-glucoside ($y = 262.31x – 9.8958$; $R^2 = 1.000$); isorhamnetin 3-*O*-rutinoside ($y = 327.42 x + 313.78$; $R^2 = 0.9991$); kaempferol 3-*O*-glucoside ($y = 190.75x – 36.158$; $R^2 = 1.000$); kaempferol 3-*O*-rutinoside ($y = 175.02x – 43.877$; $R^2 = 0.9999$); quercetin 3-*O*-glucoside ($y = 316.48x – 2.9142$; $R^2 = 1.000$); quercetin 3-*O*-rutinoside ($y = 222.79x – 243.11$; $R^2 = 0.9998$), taxifolin ($y = 478.06x + 657.33$; $R^2 = 0.9987$). The results were expressed in mg per 100 g of dry weight (dw).

2.5.2.2.2. Flavan-3-ols and galloyl derivatives

The extracts were analysed using the HPLC system described above with the following conditions thermostatted at 25 °C: (A) 2.5% acetic acid in water, (B) 2.5% acetic acid/acetonitrile (90:10, v/v) and (C) HPLC-grade acetonitrile. The elution gradient established was 0% to 100% B for 5 min, from 0 to 5% C for 35 min, from 5 to 50 % C for 5 min, isocratic 50% C for 5 min, and re-equilibration of the column, using a flow rate of 0.5 mL/min. Double online detection was carried out in the DAD using 280 nm as preferred wavelength and in a mass spectrometer (MS) connected to HPLC system via the DAD cell outlet. The MS using the same equipment described above programmed using the following settings: declustering potential (DP) -40 V, entrance potential (EP) -7 V, collision energy (CE) -20 V. EPI mode was performed in order to

obtain the fragmentation pattern of the parent ion(s) in the previous scan using the following parameters: DP -40 V, EP -10 V, CE -25 V, and collision energy spread (CES) 0 V. Spectra were recorded in negative ion mode between *m*/*z* 100 and 1400. The proanthocyanidins present in the samples were characterised according to their UV and mass spectra and retention times compared with standards when available. For the quantitative analysis of phenolic compounds, a 5-level calibration curve was obtained by injection of known concentrations (1-100 μg/ml) of different standards compounds: catechin ($y = 161.23x + 177.26$; $R^2 = 0.9992$); ellagic acid ($y = 36.81x + 257.13$; $R^2 = 0.9979$); gallic acid ($y = 298.26x – 634.14$; $R^2 = 0.9949$). The results were expressed in mg per 100g of dry weight (dw).

2.5.2.2.3. Anthocyanins

The extracts were analysed using the HPLC system described above in the conditions described by García-Marino et al., (2010). Separation was achieved on an AQUA® (Phenomenex) reverse phase C_{18} column (5 μm, 150 mm ×4.6 mm i.d) thermostatted at 35 ºC. The solvents used were: (A) 0.1% TFA in water, and (B) 100% acetonitrile. The gradient employed was: isocratic 10% B for 3 min, from 10 to 15% B for 12 min, isocratic 15% B for 5 min, from 15 to 18% B for 5 min, from 18 to 30% B for 20 min and from 30 to 35% for 5 min, at a flow rate of 0.5 mL/min. Double detection was carried out by DAD, using 520 nm as the preferred wavelength, and MS using the same equipment described above. Zero grade air served as the nebulizer gas (40 psi) and turbo gas (600 ºC) for solvent drying (50 psi). Nitrogen served as the curtain (100 psi) and collision gas (high). Both quadrupols were set at unit resolution. The ion spray voltage was set at 5000 V in the positive mode. EMS and ESI methods were used for acquisition of full scan spectra and fragmentation patterns of the precursor ions, respectively. Setting parameters used for EMS mode were: declustering potential (DP) 4 V, entrance potential (EP) 7.5 V, collision energy (CE) 10 V, and parameters for EPI mode were: DP 41V, EP 7.5 V, CE 10 V, and collision energy spread (CES) 0 V. The anthocyanins present in the samples were characterised according to their UV and mass spectra and retention times, and comparison with authentic standards when available. For quantitative analysis, a 5-level calibration curve was obtained by injection of known concentrations (50 - 0.25 μg/mL) of different standards compounds: cyaniding 3-*O*-glucoside ($y = 63027x – 153.83$; $R^2 = 0.9995$), delphinidin 3-*O*-glucoside ($y = 557274x$

+ 126.24; R^2 = 0.9999) and peonidin 3-*O*-glucoside (y = 537017x – 71.469; R^2 = 0.9997). The results were expressed in µg per 100 g of dry weight (dw).

2.6. Bioactivity evaluation

2.6.1. General

The lyophilized methanolic extracts, decoctions and infusions were redissolved in *i)* water and methanol, respectively (final concentration 2.5 mg/mL) for antioxidant activity evaluation, or *ii)* water for antitumor activity evaluation (final concentration 8 mg/mL) re-dissolved in DMSO to a final concentration of 400 µg/mL.

The results were expressed in *i)* EC_{50} values (sample concentration providing 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay) for antioxidant activity, or *ii)* GI_{50} values (sample concentration that inhibited 50% of the cell growth) for antitumor activity. Water was used as negative control, and trolox and ellipticine were used as standards in antioxidant and antitumor activity evaluation assays, respectively.

2.6.2. Antioxidant properties

DPPH radical-scavenging activity was evaluated by using an ELX800 microplate reader (Bio-Tek Instruments, Inc; Winooski, USA), and calculated as a percentage of DPPH discolouration using the formula: $[(A_{DPPH}-A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution containing the sample at 515 nm, and A_{DPPH} is the absorbance of the DPPH solution. Reducing power was evaluated by the capacity to convert Fe^{3+} into Fe^{2+}, measuring the absorbance at 690 nm in the microplate reader mentioned above. Inhibition of β-carotene bleaching was evaluated though the β-carotene/linoleate assay; the neutralization of linoleate free radicals avoids β-carotene bleaching, which is measured by the formula: β-carotene absorbance after 2 h of assay/initial absorbance) × 100. Lipid peroxidation inhibition in porcine (*Sus scrofa*) brain homogenates was evaluated by the decreasing in thiobarbituric acid reactive substances (TBARS); the colour intensity of the malondialdehyde-thiobarbituric acid (MDA-TBA) was measured by its absorbance at 532 nm; the inhibition ratio (%) was calculated using the following formula: [(A - B)/A] × 100%, where A and B were the absorbance of the control and the sample solution, respectively (Pereira et al., 2012).

2.6.3. Antitumor properties

Five human tumor cell lines were used: MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer), HCT-15 (colon carcinoma), HeLa (cervical carcinoma) and HepG2 (hepatocellular carcinoma). Cells were routinely maintained as adherent cell cultures in RPMI-1640 medium containing 10% heat-inactivated FBS (MCF-7, NCI-H460 and HCT-15) and 2 mM glutamine or in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin (HeLa and HepG2 cells), at 37 ºC, in a humidified air incubator containing 5% CO_2. Each cell line was plated at an appropriate density (7.5×10^3 cells/well for MCF-7, NCI-H460 and HCT-15 or 1.0×10^4 cells/well for HeLa and HepG2) in 96-well plates and allowed to attach for 24 h. Cells were then treated for 48 h with various extract concentrations. Following this incubation period, the adherent cells were fixed by adding cold 10% trichloroacetic acid (TCA, 100 µL) and incubated for 60 min at 4 ºC. Plates were then washed with deionized water and dried; sulforhodamine B solution (0.1% in 1% acetic acid, 100 µL) was then added to each plate well and incubated for 30 min at room temperature. Unbound SRB was removed by washing with 1% acetic acid. Plates were air dried, the bound SRB was solubilised with 10mM Tris (200 µL) and the absorbance was measured at 540 nm in the microplate reader mentioned above.

2.6.4. Hepatotoxicity

A cell culture was prepared from a freshly harvested porcine liver obtained from a local slaughter house, and it was designed as PLP2. Briefly, the liver tissues were rinsed in hank's balanced salt solution containing 100 U/mL penicillin, 100 µg/mL streptomycin and divided into 1×1 mm^3 explants. Some of these explants were placed in 25 cm^2 tissue flasks in DMEM medium supplemented with 10% fetal bovine serum, 2 mM nonessential amino acids and 100 U/mL penicillin, 100 mg/mL streptomycin and incubated at 37 ºC with a humidified atmosphere containing 5% CO_2. The medium was changed every two days. Cultivation of the cells was continued with direct monitoring every two to three days using a phase contrast microscope. Before confluence was reached, cells were subcultured and plated in 96-well plates at a density of 1.0×10^4 cells/well, and cultivated in DMEM medium with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin (Abreu et al., 2011).

2.6.5. VEGFR-2 phosphorylation inhibition

Chamaemelum nobile methanolic extract and infusion, and pure phenolic compounds (apigenin, luteolin, apigenin-7-*O*-glucuside, luteolin-7-*O*-glucuside, caffeic acid and chlorogenic acid) were assessed for VEGFR-2 inhibition activity using the Z'-LYTE-Tyr1 Peptide assay kit (Invitrogen, Cat. PV3190) according to the procedures recommended by the manufacturer (Soares et al., 2013). Briefly, assays were performed in a total of 20 µL in 384-well plates using fluorescence resonance energy transfer technology. A Tyr1 substrate (coumarin-fluorescein double-labeled peptide) at 1 µM was incubated for 1 h with 4 µg/mL VEGFR-2, 50 µM ATP and the *C. nobile* methanolic extract/infusion (400 at 6.25 µg/mL) or the pure phenolic compounds (40 at 0.04 µg/mL) at room temperature in 50 mM Hepes/NaOH (pH 7.5), 10 mM $MgCl_2$, 2 mM $MnCl_2$, 2.5 mM DTT, 0.1 mM morthovanadate, and 0.01% bovine serum albumin. The wells were incubated at 25 ºC for 1 h and 5 µL development reagents were added to each well. After a second incubation of 1 h a stop reagent was added to each well. Using a Biotek FLX800 micro-plate the fluorescence was read at 445 nm and 520 nm (excitation 400 nm), and Gen5™ Software was used for data analysis. Ginestein (Extrasynthese, Genay, France) was used as positive control.

2.6.6. Statistical analyses

For each species, three samples were used and all the assays were carried out in triplicate. The results of the chemical compostion and bioactive properties were expressed as mean values ± standard deviation (SD). The results of the antiangiogenic activity of the extracts were analyzed using a Student´s *t*-test with $\alpha = 0.05$, to determine the significant difference among the two extracts. The statistical differences in all other results, represented by letters, were obtained through one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference post hoc test with $\alpha = 0.05$. These treatments were carried out using SPSS program (v. 18.0 and 22.0).

2.6.6.1.Docking simulations using AutoDockVina and molecular Dynamics simulation

Docking simulations: the 2D structure of the compounds apigenin, apigenin-7-*O*-glucoside, luteolin and luteolin-7-*O*-glucoside was constructed using the

ACD/ChemSketch Freeware 12.0 software. Open Babel (O'Boylel et al., 2011) was used to convert compounds from 2D to 3D and saved in pdb format. A VEGFR-2 crystal structure (PDB: 2XIR) was extracted from the Protein Data Bank (PDB) (http://www.rcsb.org). The co-crystallized ligand was extracted from the PDB file, and AutoDockTools (Sanner, 2005) was used to assign polar hydrogens and Gasteiger charges to the compounds and VEGFR-2 protein. All structures were saved in PDBQT file format required to use AutoDockVina (Trott & Olson, 2010). AutodockVina was used to perform docking in an area of 30 Å by 30 Å by 30 Å, centered on the co-crystallized ligand. The docking simulations were performed on a cluster of 6 AMD Opteron 6128 8 core 2.0 GHz with using MOLA software (Abreu et al., 2010). All figures with structure representations were prepared using PyMOL (The PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC. Available at: (http://www.pymol.org/). Accessed on 03 September, 2012.

Molecular Dynamics simulation: The Protein Preparation Wizard from Maestro (Schrodinger, LLC, Portland, OR) was used to prepare ligand/VEGFR-2 complexes and then used to perform explicit solvent molecular dynamics (MD) simulations. The parallelized Desmond Molecular Dynamics System v2.2 (D. E. Shaw Research, New York, NY) and associated analysis tools, available within the Schrodinger suite (Schrodinger, LLC, Portland, OR), were used for this purpose. The protocol used is described in Mukherjee et al. (2011).

Chapter 3.

Chemical characterization and bioactive properties of the wild plants: Results and discussion

Chapter 3. Chemical characterization and bioactive properties of the wild plants: Results and discussion

3.1. *Chamaemelum nobile*

3.1.1. Nutrients composition

The results of the nutritional characterization of *Chamaemelum nobile* are shown in **Table 2**. Carbohydrates were the most abundant macronutrients, followed by proteins. Ash and fat contents were low, and the energetic contribution was 389.88 kcal/100 g dw. The main sugar found in this plant material was fructose, followed by glucose and sucrose. Trehalose was found in lower amounts. Polyunsaturated fatty acids (PUFA) predominated over saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA). The FA determined in higher percentages, were linoleic acid (C18:2n6), oleic acid (C18:1n9), α-linolenic acid (C18:3n3) and palmitic acid (C16:0). Regarding tocopherols, only α- and γ-tocopherols were found in the studied plant material. β-Carotene and lycopene were also quantified in the studied sample.

Table 2. Nutritional characterization of *Chamaemelum nobile* (mean ± SD).

Crude composition (g/100 g dw)		**Free sugars (g/100 g dw)**	
Moisture (g/100 g fw)	67.09 ± 1.02	Fructose	3.37 ± 0.24
Ash	6.43 ± 0.05	Glucose	1.57 ± 0.13
Proteins	26.63 ± 1.92	Sucrose	1.08 ± 0.08
Fat	3.12 ± 0.33	Trehalose	0.60 ± 0.02
Carbohydrates	63.83 ± 1.66	Total sugars	6.62 ± 0.31
Energy	389.88 ± 1.32		
Fatty acids (relative %)		**Tocopherols (mg/100 g dw)**	
C16:0	17.89 ± 0.16	α-Tocopherol	1.64 ± 0.02
C18:0	3.36 ± 0.03	β-Tocopherol	Nd
C18:1n9	23.22 ± 0.22	γ-Tocopherol	0.19 ± 0.01
C18:2n6	28.89 ± 0.33	δ-Tocopherol	Nd
C18:3n3	18.22 ± 0.11	Total tocopherols	1.83 ± 0.01
SFA	27.67 ± 0.19	**Pigments (mg/100 g)**	
MUFA	24.78 ± 0.27	β-carotene	0.95 ± 0.02
PUFA	47.56 ± 0.46	Lycopene	0.02 ± 0.01
PUFA/SFA	1.72 ± 0.03		
n6/n3	1.56 ± 0.01		

fw- fresh weight; dw – dry weight. Palmitic acid (C16:0); Stearic acid (C18:0); Oleic acid (C18:1n9c); Linoleic acid (C18:2n6c); α-Linolenic acid (C18:3n3). Nineteen more fatty acids were also identified and quantified (total relative percentage 8.42%; data not shown). SFA – saturated fatty acids; MUFA – monounsaturated fatty acids; PUFA – polyunsaturated fatty acids.

As far as we know this is the first report on nutritional characterization of *C. nobile*, that proved to be an equilibrated valuable herb rich in carbohydrates and proteins, and poor in fat and calories. Moreover it provides tocopherols, carotenoids, essential fatty acids (C18:2n6 and C18:3n3), and ratios PUFA/SFA and n-6/n-3 fatty acids higher than 0.45 and lower than 4.0, respectively (Guil et al., 1996), which are considered good nutritional ratios.

3.1.2. Phytochemicals in the herb and in its decoction and infusion

As *C. nobile* is mostly consumed as decoctions and infusions (aqueous extracts), a comparative study of phytochemicals present in the herb and in those preparations was performed. Oxalic, quinic, malic, citric and fumaric acids were quantified in all the extracts of *C. nobile* (**Table 3**), malic acid being the most abundant organic acid. Infusion was the preparation with the highest content in organic acids (9.07g/100 g dw), while the decoction showed the lowest amount (6.5g/100 g dw). Some of the mentioned compounds have shown bioactive properties such as the capacity to chelate metal ions of citric acid by forming bonds between the metal and its carboxyl or hydroxyl groups. Citric acid is effective in retarding the oxidative deterioration of lipids in foods and is commonly added to vegetable oils after deodorization (Hraš et al., 2000). Oxalic acid has a strong chelating ability with multivalent cations; nevertheless, it has been considered as an antinutrient due to the inhibitory effect on mineral bioavailability and to formative effect on calcium oxalate urinary stone (Kayashima & Katayama, 2002).

Table 3. Organic acids composition of *Chamaemelum nobile* (mean ± SD).

Organic acid	Herb	Decoction	Infusion
Oxalic acid	2.02 ± 0.06^{a}	1.74 ± 0.21^{b}	$1.99 \pm 0.13^{b,a}$
Quinic acid	1.74 ± 0.13^{b}	1.40 ± 0.04^{b}	2.56 ± 0.17^{a}
Malic acid	3.02 ± 0.07^{a}	2.21 ± 0.19^{b}	3.06 ± 0.05^{a}
Citric acid	1.33 ± 0.01^{a}	1.23 ± 0.16^{a}	1.46 ± 0.24^{a}
Fumaric acid	0.02 ± 0.00^{a}	0.01 ± 0.00^{b}	0.01 ± 0.00^{b}
Total (g/100 g)	8.14 ± 0.28^{b}	6.58 ± 0.28^{c}	9.07 ± 0.01^{a}

In each row different letters mean significant differences ($p<0.05$)

The main phenolic compounds found in *C. nobile* herbal material and in its decoction and infusion were flavonoids (flavonols and flavones), phenolic acids and derivatives (**Table 4 and 5**).

Table 4. Retention time (Rt), wavelengths of maximum absorption in the UV-vis region (λ_{max}), pseudomolecular and MS^2 fragment ions (in brackets, relative abundances) and tentative identification of the phenolic compounds of *Chamaemelum nobile*.

Peak	Rt (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification
1	5.11	326	353	191(100), 179(69), 161(7), 135(51)	3-*O*-Caffeolyquinic acid
2	5.65	320	515	353(11), 341(6), 323(100), 191(61), 179(6), 161(18), 135*	5-*O*-Caffeoylquinic acid-hexoside
3	6.15	262,294	153	109(100)	Protocatechuic acid
4	6.44	326	469	307(8), 179(100),161(37), 135(78)	Caffeoyl-hexoside-methylglutarate
5	7.79	326	353	191(100), 179(11), 173(9), 161(28), 135(8)	5-*O*-caffeolyquinic acid
6	9.62	312	453	291*, 163(100), 145(9), 119(51)	*p*-coumaroyl-hexoside-methylglutarate
7	10.48	332	593	593(100), 473(18), 383(6), 353(12)	Apigenin 6-*C*-glucose-8-*C*-glucose
8	11.05	316	453	163(100), 145(8),119(42)	*p*-coumaroyl–hexoside-metylglutarate
9	11.64	328	469	469(100), 307*, 179(6),161(19), 135(3)	Caffeoyl-hexoside-methylglutarate
10	16.19	320	445	445(100), 427(58), 265(15), 179(8)	Caffeic acid derivative
11	16.53	356	479	317(100)	Myricetin 3-*O*-glucoside
12	17.15	344	621	487(3), 351(100), 269(14)	Apigenin derivative
13	17.32	320	677	677(100), 515(28), 497(16), 353(65), 335(23), 191 (12), 179(8), 135(3)	1,3,5-*O*-tricaffeoylquinic acid
14	18.26	356	477	301(100)	Quercetin 3-*O*-glucuronide
15	18.52	342	607	269(100)	Apigenin *O*-glucuronylhexoside
16	18.71	350	579	579(100), 417(26), 285(53)	Kaempferol *O*-pentosylhexoside
17	19.01	350	593	285(100)	Luteolin *O*-rutinoside
18	19.12	344	607	545(5), 337(27), 269(100)	Apigenin derivative
19	19.63	350	579	579(100), 447(4), 285(16)	Luteolin *O*-pentosylhexoside
20	19.70	350	461	285(100)	Luteolin *O*-glucuronide
21	20.06	344	593	593(100), 447(5), 285(17)	Luteolin *O*-rhamnosylhexoside
22	20.11	332	515	515(100), 353(54), 335(16),299(3),203(3),191(16), 179(27), 173(44),135(11)	3,4-*O*-Dicaffeoylquinic acid
23	21.14	350	447	285(100)	Luteolin *O*-hexoside
24	21.75	328	515	353(100), 335(6), 191(85), 179(42), 173(11), 135(16)	3,5-*O*-Dicaffeoylquinic acid
25	23.59	370	549	505(100), 301(74)	Quercetin 7-*O*-malonylhexoside
26[a]	24.26	318sh ,348	505	301(100)	Quercetin *O*-acetylhexoside
26b	24.23	338	445	269(100)	Apigenin *O*-glucuronide
27	24.52	338	649	605*, 587(5), 515(5), 427(5), 379(29), 361(3), 311(4), 269(100)	Apigenin derivative
28	25.05	332	489	285(100)	Luteolin *O*-acetylhexoside
29	25.27	334	445	269(100)	Apigenin *O*-glucuronide
30	27.18	318sh,358	519	315(100)	Isorhamnetin *O*-acetylhexoside

*abundance ≤ 2; 26b-only found in decoction sample.

Table 5. Quantification of phenolic compounds in *Chamaemelum nobile* (mean ± SD).

Peak	Herb	Decoction	Infusion
1	0.07 ± 0.01	nd	0.08 ± 0.01
2	0.21 ± 0.01	0.04 ± 0.01	0.15 ± 0.02
3	0.04 ± 0.01	0.06 ± 0.01	0.10 ± 0.0
4	0.07 ± 0.01	nd	0.05 ± 0.00
5	1.70 ± 0.00	nd	1.52 ± 0.04
6	tr	nd	tr
7	0.04 ± 0.00	0.03 ± 0.00	0.01 ± 0.00
8	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00
9	0.01 ± 0.00	nd	0.01 ± 0.00
10	0.01 ± 0.00	nd	0.02 ± 0.00
11	0.01 ± 0.00	nd	0.01 ± 0.00
12	0.11 ± 0.01	0.17 ± 0.01	0.15 ± 0.01
13	0.17 ± 0.04	nd	0.13 ± 0.00
14	0.10 ± 0.00	nd	0.05 ± 0.01
15	0.06 ± 0.01	0.02 ± 0.00	0.02 ± 0.00
16	0.04 ± 0.01	0.02 ± 0.00	nd
17	0.42 ±0.03	nd	0.11 ± 0.00
18	0.15 ± 0.01	0.50 ± 0.05	0.07 ± 0.01
19	0.31 ± 0.07	0.18 ± 0.01	0.13 ± 0.00
20	0.19 ± 0.04	0.07 ± 0.01	0.16 ± 0.00
21	0.37 ± 0.02	0.12 ± 0.01	nd
22	0.02 ± 0.00	nd	0.01 ± 0.00
23	0.37 ± 0.06	0.06 ± 0.00	0.39 ± 0.02
24	0.37 ± 0.07	nd	0.06 ± 0.02
25	0.11 ± 0.01	0.01 ± 0.00	0.03 ± 0.00
26a	0.05 ± 0.01	nd	0.04 ± 0.01
26b	nd	0.06 ± 0.01	nd
27	0.88 ± 0.05	0.29 ± 0.03	0.76 ± 0.02
28	0.46 ± 0.07	nd	0.24 ± 0.00
29	0.12 ± 0.01	nd	0.10 ± 0.0
30	0.02 ± 0.00	nd	0.11 ± 0.00
Total flavonol derivatives (g/100g)	0.34 ± 0.03^{a}	0.02 ± 0.00^{c}	0.13 ± 0.01^{b}
Total flavone derivatives (g/100g)	3.48 ± 0.25^{a}	1.51 ± 0.02^{c}	2.18 ± 0.03^{b}
Total caffeoylquinic acids (g/100g)	2.54 ± 0.13^{a}	0.04 ± 0.01^{c}	1.94 ± 0.07^{b}
Total phenolic acid derivatives (g/100g)	0.15 ± 0.02^{b}	0.08 ± 0.01^{c}	0.19 ± 0.00^{a}
Total phenolic compounds (g/100g)	6.51 ± 0.43^{a}	1.65 ± 0.03^{c}	4.44 ± 0.11^{b}

In each row different letters mean significant differences ($p<0.05$). tr- traces; nd-not detected.

In general, all the preparations revealed the same chromatographic profile, exemplified in **Figura 7A** for the herbal sample.

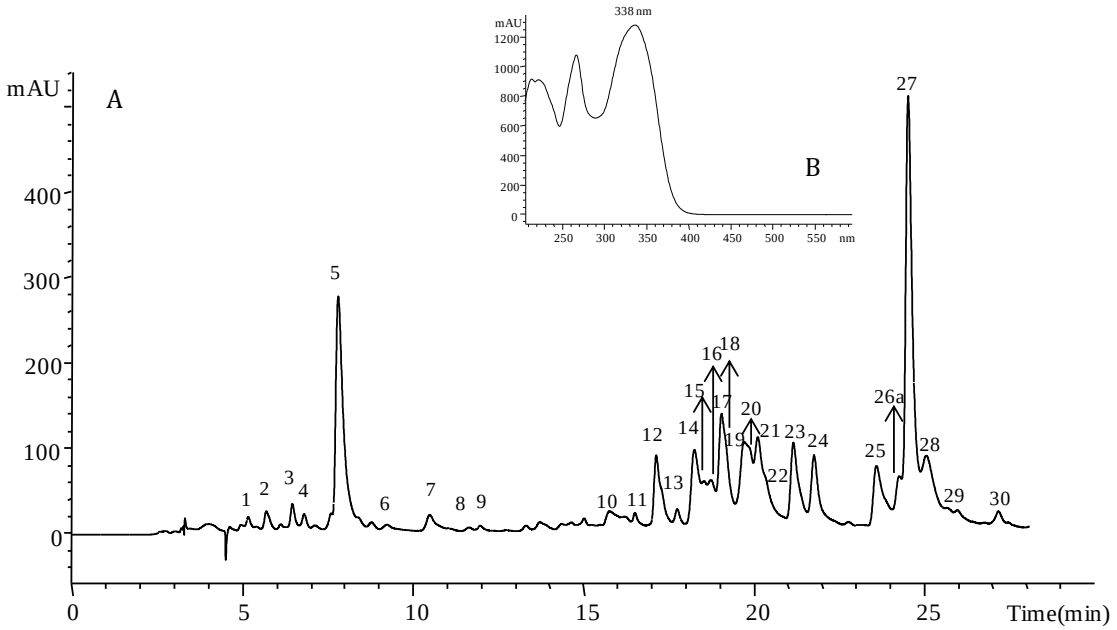


Figure 7. HPLC chromatogram of the phenolic compounds of *Chamaemelum nobile* recorded at 370 nm (A) and UV spectra of peak 27 (B).

Up to thirty-one phenolic compounds, including a phenolic acid, eleven hydroxycinnamic acid derivatives and nineteen flavonoids were detected in the *C. nobile* preparations (**Table 4**). Peak 3 was identified as protocatechuic acid by comparison of its UV spectrum and retention time with a commercial standard. Six hydroxycinnamic acid derivatives (peaks 1, 2, 5, 13, 22 and 24) showed UV spectra with maximum wavelengths around 320-330 nm and yielded fragment ions at *m/z* 191 (deprotonated quinic acid) and 179 (deprotonated caffeic acid) in their MS^2 mass spectra, which together with their pseudo molecular ions $[M-H]^-$ at *m/z* 353, 515 and 677, allowed their identification as quinic acid derivatives containing one, two or three caffeic acid moieties, respectively. Peak assignments of the different caffeoylquinic acid isomers were made using the recommended IUPAC numbering system (IUPAC, 1976) as also the hierarchical keys previously developed by Clifford et al., 2003 and 2005. The majority peak 5 ($[M-H]^-$ at *m/z* 353) was positively identified as 5-*O*-caffeoylquinic acid by comparison with an authentic standard. Peak 1 ($[M-H]^-$ at *m/z* 353) was identified as 3-*O*-caffeoylquinic acid, yielding the base peak at *m/z* 191 and the ion at *m/z* 179 with an intensity >50% base peak. Similar fragmentation pattern was reported by Clifford et al. (2003, 2005) as characteristic to distinguish 3-acylchlorogenic acids. Peaks 2, 22, 24 ($[M-H]^-$ at *m/z* 515) could be assigned as dicaffeoylquinic acids. Peaks 22 and 24 were assigned to 3,4-*O*- and 3,5-*O*-dicaffeoylquinic acids, respectively, based on their elution order, fragmentation

pattern and relative abundances (Clifford et al., 2003, 2005). MS^2 fragmentation of peak 22 yielded the formation of relatively intense signals corresponding "dehydrated" fragments at *m/z* 335 [caffeoylquinic acid –H-H_2O] $^-$ and *m/z* 173 [quinic acid-H-H_2O]$^-$, characteristic of 4-acyl-caffeoylquinic acids. Furthermore, according to Clifford et al. (2005), the intensity of signal at *m/z* 335 (16% of base peak) is more intense than in 4,5-*O*-dicaffeoylquinic (barely detectable, <5% of base peak). These observations allowed assigning peak 22 as 3,4-*O*-dicaffeoylquinic acid. The fragmentation pattern for 3,5-*O*-dicaffeoylquinic (peak 24) acid was similar to the one previously reported by Clifford et al., 2005. MS^2 base peak was at *m/z* 353, produced by the loss of one of the caffeoyl moieties [M-H-caffeoyl]$^-$, and subsequent fragmentation of this ion yielded the same fragments as a 5-caffeoylquinic acid at *m/z* 191, 179 and 135, although in this case with a comparatively more intense signal at *m/z* 179 [caffeic acid-H]$^-$ (<50% base peak).

Peak 2 showed the same pseudomolecular ion as peaks 22 and 24 but eluted much earlier than those dicaffeoylquinic acids and also than 5-*O*-caffeoylquinic acid. In addition to the fragments characteristic of a caffeoylquinic acid, the MS^2 spectrum of this compound also produced fragments at *m/z* 353 ([M-H-162]-, loss of a hexose residue) and 341 ([M-H-174]-, loss of a quinic acid moiety) and its "dehydrated" form at *m/z* 323. This fragmentation pattern pointed to a glycosylated chlorogenic acid, which could correspond to 1- or 5-caffeoylquinic-hexoside, according to the mass spectra characteristics and intensities of MS^2 fragment ions as reported by Clifford et al., 2007. The fact that 5-*O*-caffeoylquinic acid was the majority compound in the sample and 1-*O*-caffeoylquinic acid was not detected, permitted its tentative identification as 5-*O*-caffeoylquinic acid-hexoside, identity that was coherent with its early elution (greater polarity) compared with its parent aglycone. Peak 13 was identified as tricaffeoylquinic acid according to its pseudomolecular ion [M-H]$^-$ at *m/z* 677 and diagnostic MS^2 fragments at *m/z* 515 (loss of the first caffeoyl), *m/z* 353 (loss of the second caffeoyl), *m/z* 191 (loss of the third caffeoyl to give quinic acid ion), *m/z* 179 (caffeic acid ion). The signal observed at *m/z* 497 can be interpreted by the loss of a water molecule from the ion at *m/z* 515. According to the relative intensities of different tricaffeoylquinic acid isomers reported by Lin & Harnly (2008), this peak could be assigned as 1,3,5-*O*-tricaffeoylquinic acid. Peaks 4, 9 and 10, with UV spectra similar to caffeic acid with λ_{max} around 326 nm, were also assigned to caffeic acid derivatives. All of them presented an MS^2 fragment at *m/z* 179 ([caffeic acid-H]$^-$). Peaks 4 and 9 also presented a fragment at *m/z* 307 (-162 mu, loss of a hexose moiety), and the formation of the ion at *m/z* 179 could be produced by the loss of 162 +

128 mu (loss of hexose + methyl-glutarate residues), so that they were tentatively identified as caffeoyl-hexoside-methylglutarate. Although they could not be fully identified, these compounds could be attributed as derived from the *cis* and *trans* isomers of caffeic acid. The MS^2 analysis of peak 10 yielded signals at *m/z* 427 ($[M-H_2O]^-$) and *m/z* 265 ($[M-18-162]^-$) pointing out to the presence of a hexose, although no further conclusions could be made bout its definite identity.

Peaks 6 and 8 ($[M-H]^-$ at *m/z* 453) presented a molecular weight 16 units lower than peaks 4 and 9 but a similar fragmentation pattern, indicating that they could be the corresponding coumaroyl derivatives; this assumption was also supported by the formation of the MS^2 fragment ion at *m/z* 163 ($[coumaric\ acid-H]^-$). Thus, they were tentatively identified as two *p*-coumaroyl-hexoside-methylglutarate. Their later elution (lower polarity) compared with the caffeoyl counterparts (peaks 4 and 9) was also coherent with this identity; similarly, they could be speculated as the respective *cis* and *trans* isomers. Flavones were the most abundant flavonoids present in the analysed samples (**Table 5**). Peaks 7, 12, 15, 18, 26b, 27 and 29 were identified as apigenin derivatives, according to their UV and mass spectra characteristics (**Figure 7A**). Peak 7 presented a pseudo molecular ion $[M-H]^-$ at *m/z* 593, releasing three MS^2 fragments ions at *m/z* 473 and 383, corresponding to loss of 120 and 90 amu, characteristic of *C*-hexosyl flavones, and at *m/z* 353 that might correspond to the apigenin aglycone bearing some sugar residues [apigenin+83 mu] (Ferreres et al., 2003). The fact that no relevant fragment derived from the loss of a complete hexosyl residue (-162 mu) was detected, suggested that both sugars were *C*-attached, which allowed a tentative identification of the compound as apigenin 6-*C*-glucose-8-*C*-glucose. Peaks 15, 26b and 29 presented pseudo molecular ions $[M-H]^-$ at *m/z* 607 and 445 releasing a MS^2 fragment ion at *m/z* 269 ($[M-176-162]^-$ and $[M-176]^-$, corresponding to the loss of glucuronyl-hexoside and glucuronyl moieties, respectively), being tentatively identified as apigenin *O*-glucuronyl-hexoside (peak 15) and apigenin *O*-glucuronides (peaks 26b and 29). Peaks 12, 18 and 27 presented pseudomolecular ions $[M-H]^-$ at *m/z* 621, 607 and 649, respectively, that release an MS^2 fragment at *m/z* 269 (apigenin). They all presented a similar fragmentation pattern, with a loss of 270 mu, and peaks 18 and 27 also have a fragment ion at $[M-338]^-$, that could correspond to glucuronyl-hexoside or feruloyl-hexoside [M-176-162]. Moreover, peak 27 presents a difference of 42 mu relatively to peak 18 that may be due to an acyl group. Peaks 12 and 18 also presented a difference of 14mu that could correspond to a methyl group. However, the fragmentation patterns of these compounds did not allow us to

conclude further about their chemical structure, but due to the UV spectra (**Figure 7B**) and the fragmentation mentioned above they were just associated to unknown apigenin derivatives. Peaks 17, 19, 20, 21, 23 and 28 were identified as luteolin derivatives. Peaks 17, 20, 23 and 28 presented pseudomolecular ions [M-H]- at *m/z* 593, 461, 447 and 489 releasing a common MS^2 fragment at *m/z* 285 ($[M-308]^-$, $[M-176]^-$, $[M-162]^-$ and [M-42-162], associated to the loss of rutinosyl, glucuronyl, hexosyl and acetylhexoside moieties, respectively). They were tentatively identified as luteolin *O*-rutinoside, luteolin *O*-glucuronide, luteolin *O*-hexoside (the retention time is different from luteolin 7-*O*-hexoside) and luteolin *O*-acetylhexoside. Peaks 19 and 21 showed pseudomolecular ions $[M-H]^-$ at *m/z* 579 and 593, both releasing two MS^2 fragments at *m/z* 447 ($[M-132]^-$ and $[M-146]^-$ loss of pentosyl and rhamnosyl moieties, respectively) and 285 ($[M-162]^-$, loss of a hexosyl moiety), being tentatively identified as luteolin *O*-pentosyl-hexoside and luteolin *O*-rhamnosyl-hexoside.

Flavonols (peaks 11, 14, 16, 25, 26a and 30) were also found in the studied samples (**Tables 4** and **5**). Peak 11 presented a pseudomolecular ion $[M-H]^-$ at *m/z* 479, releasing an MS^2 fragment at *m/z* 317 ($[M-H-162]^-$, loss of an hexosyl moiety), corresponding to myricetrin, which allowed a tentative identification of the compound as myricetin 3-*O*-glucoside, as confirmed by comparison with an authentic standard. Peak 16 showed a pseudomolecular ion $[M-H]^-$ at *m/z* 579, releasing two MS^2 fragments at *m/z* 417 ($[M-H-162]^-$, loss of a hexosyl moiety) and 285 (kaempferol; $[M-H-162-132]^-$, loss of a further pentosyl moiety), being tentatively identified as kaempferol pentosyl-glucoside the two glycosyl residues are assumed to constitute a disaccharide taking into account that the are lost successively and no alternatively, with no fragment corresponding to a kaempferol-pentoside being released. Peaks 14, 25 and 26a corresponded to quercetin derivatives. Peak 14 presented a pseudomolecular ion $[M-H]^-$ at m/z 477, releasing an MS^2 fragment at *m/z* 301 ($[M-H-176]^-$, loss of a glucuronyl moiety); this compound was identified as quercetin 3-*O*-glucuronide, by comparison with a standard isolated in our laboratory (Dueñas et al., 2008). Peak 25 presented a pseudomolecular ion $[M-H]^-$ at *m/z* 549, releasing a MS^2 fragments at *m/z* 301 ($[M-H-162-86]^-$, loss of a malonylhexoside moiety). The UV/Vis spectra have long been used for structural analysis of flavonoids. The typical flavonoid spectrum consists of two maxima in the range 240–285 nm (Band II), and 300–550 nm (Band I), which is more specific and useful for obtaining information regarding identification. The position and relative intensities of these maxima yield information on the nature of the flavonoid and its hydroxylation and substitution patterns (Santos-Buelga

et al., 2003). It is known that the introduction of a glycoside on the hydroxyls at positions 7, 3´ or 4´ has no effect on wavelength maxima or the spectrum shape in relation to the aglycone. Thus, quercetin 7-*O*-glycosides would have λ_{max} in Band I around 370 nm, while quercetin 3-*O*-glycosides are hypsochromically shifted to around 354 nm. Since peak 25 presented λ_{max} at 370 nm it was tentatively identified as quercetin 7-*O*-malonylhexoside. Peak 26a presented a pseudomolecular ion $[M-H]^-$ at *m/z* 505 releasing an MS^2 fragment at *m/z* 301 (quercetin; $[M-H-42-162]^-$, loss of an acetylhexoside moiety), and was tentatively identified as quercetin *O*-acetylhexoside. Peak 30 presented a pseudomolecular ion [M-H]- at *m/z* 519 releasing a MS^2 fragment at *m/z* 315 (isorhmanetin; $[M-H-42-162]^-$, loss of an acetylhexoside moiety), so that it was tentatively identified as isorhamnetin *O*-acetylhexoside. The amounts of the phenolic compounds found varied among the different preparations and some compounds disappeared, mostly in decoctions (**Table 5**). Peak 26a, quercetin *O*-acetylhexoside that appeared in the plant material and infusion preparation, was not present in the decoction preparation, being detected at the same retention time another compound (peak 26b) associated to an apigenin glucuronide. Tschan, 1996 and Carnat et al., 2004, reported in *Chamaemelum nobile* the presence of chamaemeloside (i.e., apigenin 7-glucoside-6"-(3"'-hydroxy-3"'-methyl-glutarate), but this compound was not detected in the sample of roman camomile studied herein. Carnat et al., 2004, did not report in their study the presence in roman camomile of some flavonols described in the present work, such as the quercetin, kaempferol and isorhamnetin derivatives, but they only report flavones and phenolic acids also detected in this study.

3.1.3. Antioxidant and antitumor activity

The antioxidant properties were evaluated by four different tests as there is no universal method that can measure the antioxidant capacity of all samples accurately and quantitatively: DPPH radical scavenging capacity, reducing power and inhibition of lipid peroxidation using β-carotene–linoleate model system in liposomes and TBARS assay in brain homogenates.

Table 6. Antioxidant and antitumor activities of *Chamaemelum nobile* (mean ± SD).

	Herb	**Decoction**	**Infusion**	**Standard***
Antioxidant activity (EC50 values, µg/mL)				
DPPH scavenging activity	621.64 ± 6.84^{b}	1477.30 ± 71.99^{a}	408.46 ± 11.34^{c}	43.03 ± 1.71
Reducing power	294.90 ± 10.97^{b}	532.62 ± 8.75^{a}	258.89 ± 3.36^{b}	29.62 ± 3.15
β-carotene bleaching inhibition	443.32 ± 1.47^{c}	680.54 ± 69.06^{b}	1219.64 ± 31.56^{a}	2.63 ± 0.14
TBARS inhibition	82.33 ± 11.53^{c}	268.55 ± 5.32^{a}	120.41 ± 4.24^{b}	3.73 ± 1.90
Antitumor activity (GI_{50} values, µg/mL)				
MCF-7 (breast carcinoma)	82.52±4.57^{b}	>400	247.95±5.56^{a}	0.91±0.04
NCI-H460 (non-small cell lung cancer)	82.75±8.14^{b}	>400	226.65±7.42^{a}	1.42±0.00
HCT-15 (colon carcinoma)	91.23±3.13^{b}	>400	150.24±5.47^{a}	1.91±0.06
HeLa (cervical carcinoma)	85.01±6.39^{b}	>400	233.06±7.36^{a}	1.14±0.21
HepG2 (hepatocellular carcinoma)	168.40±2.23^{b}	>400	250.30±0.55^{a}	3.22±0.67
PLP2	>400	>400	>400	>32.5

*Trolox and ellipticine for antioxidant and antitumor activity assays, respectively. EC_{50} values correspond to the sample concentration achieving 50% of antioxidant activity or 0.5 of absorbance in reducing power assay. GI_{50} values correspond to the sample concentration achieving 50% of growth inhibition in human tumor cell lines or in liver primary culture PLP2. In each row different letters mean significant differences ($p<0.05$).

As it can be observed in **Table 6**, herbal sample gave the highest β-carotene bleaching activity and lipid peroxidation inhibition (lowest EC_{50} values, **Table 6**) which can be related to its higher content in phenolic compounds **(Table 5**), while infusion showed the highest DPPH scavenging activity (**Table 5**) which may be related to their higher levels in organic acids (**Table 3**). Both samples showed similar reducing power (**Table 6**). The decoction presented the lowest antioxidant properties, probably due to its lower content in phenolic compounds and organic acids.

The effects of *C. nobile* extract, decoction and infusion on the growth of five human tumor cell lines (MCF-7, NCI-H460, HCT-15, HeLa and HepG2), represented as the concentrations that caused 50% of cell growth inhibition (GI_{50}), are also summarized in **Table 6**. The plant material extract was more potent than the infusion sample in all the tested cell lines, presenting GI_{50} values that ranged from 82.52 to 168.40 µg/mL for the MCF-7 and HepG2 cells, respectively. Decoction preparation had no antitumor effects at the maximal concentration used (400 µg/mL), which could indicate that these effects might be related to compounds (including phenolic compounds) that are not extracted or affected by the decoction. Nevertheless, none of the *C. nobile* preparation showed

hepatotoxicity in the porcine liver primary cell culture (non-tumor cells; PLP2) (**Table 6**). Overall, *C. nobile* is an equilibrated valuable species rich in carbohydrates and proteins, and poor in fat, providing tocopherols, carotenoids and essential fatty acids (C18:2n6 and C18:3n3). Moreover, the herb and its infusion are a source of phenolic compounds and organic acids that showed antioxidant and antitumor activities, without hepatotoxicity. Some bioactive compounds are affected by in *C. nobile* decoction, leading to a lower antioxidant potential and absence of antitumor potential. The plant bioactivity could be explored in the medicine, food, and cosmetic industries.

3.1.4. Antiangiogenic activity of the extracts and phenolic compounds

According to previous studies of the authors, Roman chamomile is an equilibrated valuable species rich in carbohydrates and proteins, and poor in fat, providing tocopherols, carotenoids and essential fatty acids (C18:2n6 and C18:3n3). Moreover, the herb and its infusion are a source of phenolic compounds and organic acids with a high bioactive potential (Guimarães et al., 2013). Herein, methanolic extract, infusion and phenolic compounds of Roman chamomile were evaluated for their ability to interact with the VEGFR-2 tyrosine kinase domain, using an enzymatic fluorescence resonance energy transfer (FRET-based assay). The results are given in **Table 7**.

Table 7. VEGFR-2 phosphorylation inhibition activity of *Chamaemelum nobile* extracts and phenolic compounds (mean ± SD).

Chamaemelum nobile	VEGFR-2 IC_{50}, µg/mL
Methanolic extract	269.26 ± 8.74
Infusion	301.09 ± 13.07
t-Students test; *p*-value	<0.001
Phenolic compound	VEGFR-2 IC_{50}, µM
Luteolin	2.10 ± 0.32c,*
Apigenin	4.78 ± 0.27b
Apigenin-7-O-glucoside	44.47 ± 3.96a
Luteolin-7-O-glucoside	>40
Caffeic acid	>40
Chlorogenic acid	>40
Genistein	1.04 ± 0.06

IC_{50}- concentration that provided 50% of VEGFR-2 inhibition. *Different letters mean significant differences between compounds (p<0.05).

The methanolic extract showed a lower IC_{50} value than the infusion, 269 and 301 μg/mL, respectively. These results are in agreement with the higher phenolic compounds amount, antioxidant and antitumor activities also previously reported for the methanolic extract. (Guimarães et al., 2013). Regarding individual molecules, apigenin, apigenin-7-*O*-glucoside, caffeic acid, chlorogenic acid, luteolin and luteolin-7-*O*-glucoside were chosen because these compounds were the ones used to quantify all the phenolic compounds identified in Roman chamomile (Guimarães et al., 2013). Phenolic acids (caffeic and chlorogenic acids) and luteolin-7-*O*-glucoside did not show VEGFR-2 phosphorylation inhibition activity (IC_{50} values higher than 40 μg/mL), whereas apigenin-7-*O*-glucoside gave VEGFR-2 phosphorylation inhibition activity with IC_{50} value = 19.21 μg/mL. A drastically increase in the VEGFR-2 phosphorylation inhibition activity was observed for the corresponding aglycones (compounds without the glycosyl group) of the mentioned flavonoids: luteolin and apigenin (IC_{50} values = 0.60 and 1.29 μg/mL, respectively). The active concentrations, corresponding to the last IC_{50} values, are easily provided by the Roman chamomile infusion, which contains 8.42 μg/mL and 9.28 μg/mL of luteolin and apigenin derivatives (compounds with glycosyl groups: luteolin7-*O*-glucoside and apigenin-7-*O*-glucoside), respectively (values calculated from the ones reported previously by the authors and taking into account the extraction yields) (Guimarães et al., 2013). It should be highlighted that the methanolic extract prepared from the herb would provide even higher amounts of those derivatives (21.31 and 13.50 μg/mL, respectively, Guimarães et al., 2013. The possible VEGFR-2 phosphorylation inhibition mechanism of luteolin, apigenin and apigenin-7-*O*-glucoside (**Figure 8**) was predicted using docking tools.

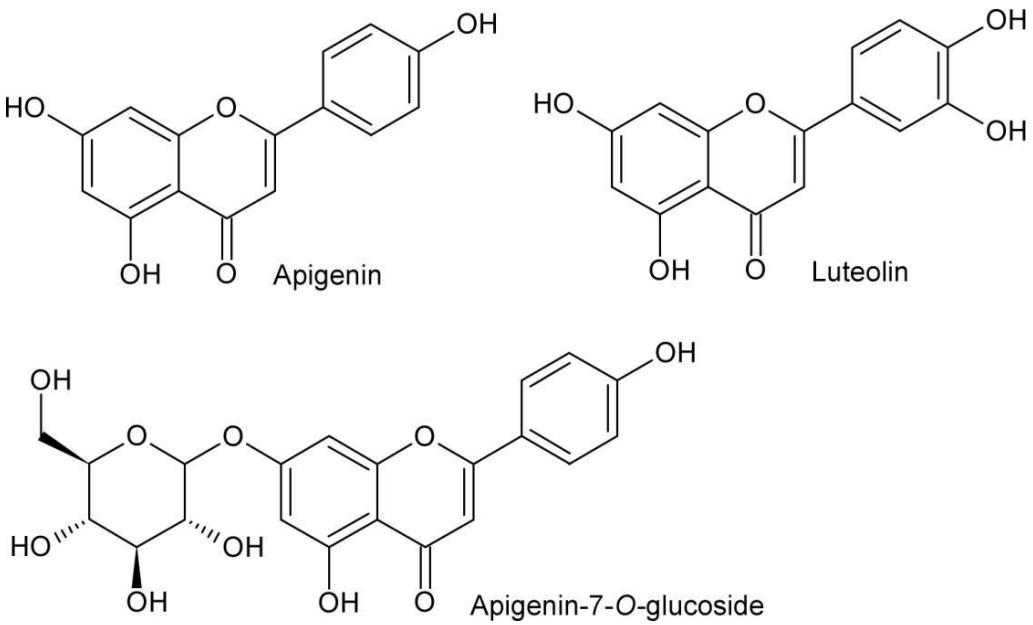


Figure 8. Chemical structures of luteolin, apigenin and apigenin-7-*O*-glucoside.

A careful analysis of the predicted docking poses showed that apigenin and luteolin probably interacts with VEGFR-2 ATP binding site with a similar binding pose, stabilized by three predicted hydrogen bonds (**Figure 8**): one H-bond between the CYS919 backbone and the carbonyl group in position 3 of the benzopyrone moiety; a second H-bond between CYS919 backbone and the hydroxy group in position 5 of the benzopyrone moiety; and a third H-bond between the amino group of LYS868 side chain and the hydroxy group in position 4 of the benzene ring. The higher VEGFR-2 phosphorylation inhibition capacity of luteolin compared to apigenin can probably be explained with a better occupation of the ATP binding site, accomplished by the lutein extra hydroxyl group occupation of a small pocket located inside the **Figure 9**.

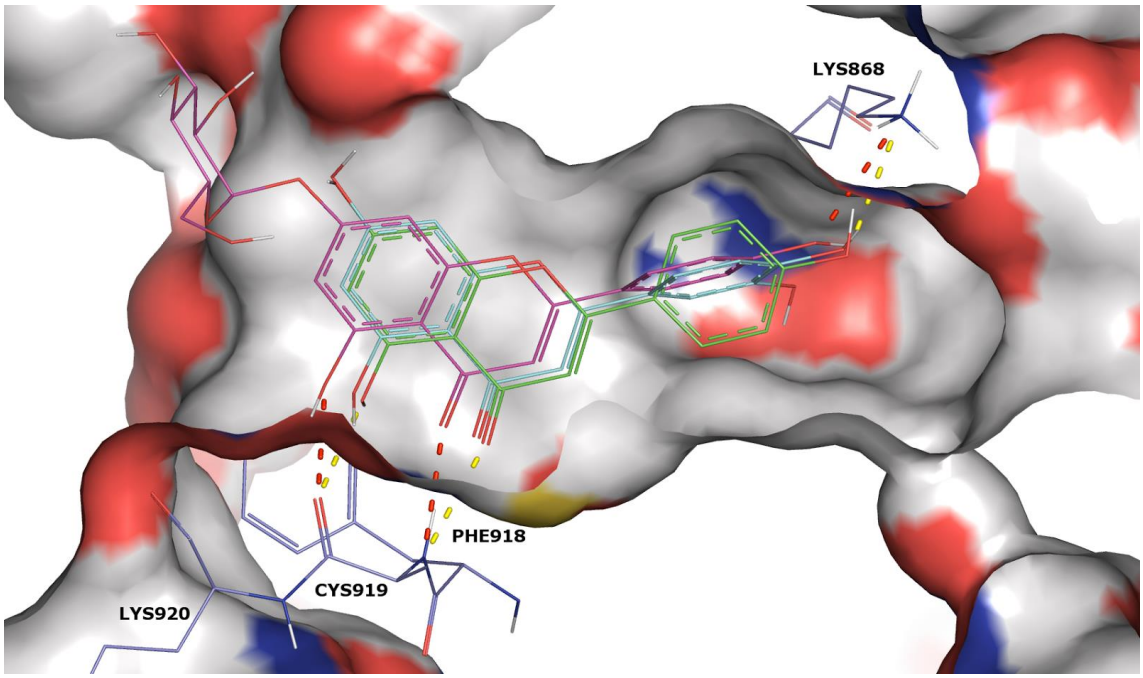


Figure 9. Surface representation of VEGFR-2 ATP binding site docked with apigenin (green line), luteolin (blue line) and apigenin-7-*O*-glucoside (magenta line). Apigenin and luteolin hydrogen bonds are represented at yellow dash, and apigenin-7-*O*-glucoside hydrogen bonds at red dash.

Furthermore, comparing the docking poses of apigenin and apigenin-7-*O*-glucoside, it was possible to observe that the presence of the glucoside moiety shifts the compound slightly away from the ATP binding site. This shift probably weakens the described H-bonds, explaining the lower VEGFR-2 phosphorylation inhibition capacity of apigenin-7-*O*-glucoside. Moreover, the inability of AutodockVina to predict a binding pose of luteolin-7-*O*-glucoside similar to luteolin, apigenin and apigenin-7-*O*-glucoside, seems to indicate that luteolin-7-*O*-glucoside probably cannot interact with the ATP binding site. This was experimentally proved by the high IC_{50} value obtained in the enzymatic assay (>100 µM). MD (Molecular Dynamics) simulation were performed using the most active compounds, luteolin and apigenin, to verify if both predicted docking poses remain stable in a more physiologically relevant setting. The docking posed of both complexes were the starting points for 5 ns MD simulations, and the overall stability of each MD simulation was evaluated by plotting the receptor backbone (VEGFR-2) and ligands RMSD (Root Mean Square Deviation) as a function of time (**Figure 10**).

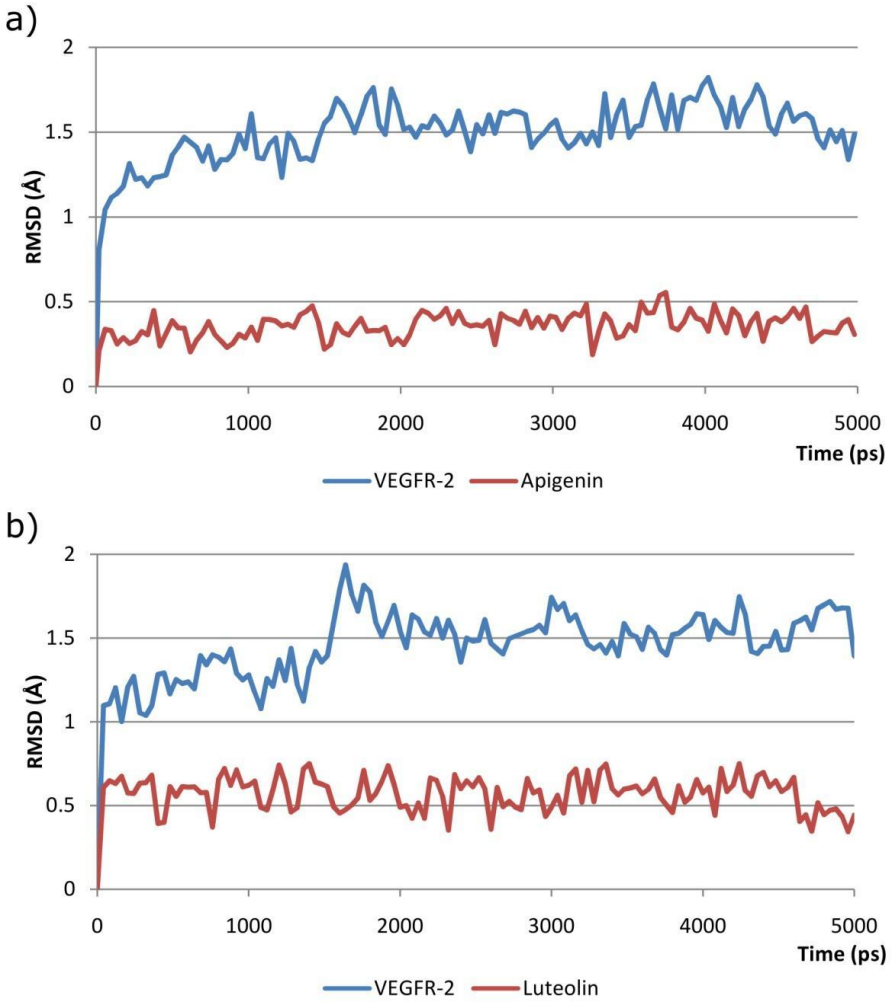


Figure 10. RMSD values obtained during the 5 ns MD simulation timeframe for: a) VEGFR-2/Apigenin and b)VEGFR-2/Luteolin complexes.

After small adjustments in the first ns of the MD simulation, both apigenin and luteolin structures remained stable thought-out the duration of the MD simulation with average RMSD of 0.37 and 0.57 Å, respectively (**Figure 10)**. This is an indication that the predicted docking pose is reliable and is probably close to the experimental VEGFR-2 binding pose. In both MD simulations, the RMSD values for the VEGFR-2 backbone structure was also analyzed and it was observed that, after a normal adjustment of around 2 ns, the RMSD values also remained stable thought-out the rest of the MD simulation. This is the expected MD simulation behavior of the protein backbone indicating that the VEGFR-2 structure used is suitable for this type of molecular modeling studies.

In general the MD simulations performed give us further assurance that the predicted docking pose probably corresponds closely to the experimental binding pose although

this can only be completely established by the elucidation of the VEGFR-2/apigenin or VEGFR2/luteolin complex structures, usually performed by X-ray crystallography.

The antiangiogenic effect of apigenin on tumor cells was already reported but related to a reduction in the expression of VEGF (Osada et al., 2004) and not with an inhibition of VEGFR activity, such it was demonstrated in the present work. Regarding luteolin, as far as we know this is the first report on antiangiogenic activity, being only reported its anticarcinogenic effects mainly by induction of apoptosis and cell cycle arrest by action on critical molecular targets for cell survival such as p53, p21, cyclin dependent kinases and caspases in liver (Stagos et al., 2012) and non-small cell lung (Cai et al., 2011) cancer cells.

3.2. *Matricaria recutita*

3.2.1. Antioxidant and antitumor activity

The antioxidant activity was determined by free radicals scavenging activity, reducing power and inhibition of lipid peroxidation in brain cell homogenates, the antitumor potential was tested in human tumor cell lines (breast, lung, colon, cervical and hepatocellular carcinomas), and the hepatotoxicity was evaluated using a porcine liver primary cell culture. The results are shown in **Table 8**.

Table 8. Antioxidant and antitumor activities, and hepatotoxicity of wild *Matricaria recutita* (mean ± SD)

	Infusion	Decoction	Plant methanol extract*	Positive control**
Extraction yield (%)	16.25 ± 0.59	19.37 ± 1.09	16.09 ± 0.77	-
Antioxidant activity (EC_{50} values, µg/mL)				
DPPH scavenging activity	394.97 ± 44.31^{b}	344.02 ± 18.65^{b}	800.36 ± 49.09^{a}	43.03 ± 1.71
Reducing power	316.61 ± 2.46^{a}	318.75 ± 3.01^{a}	232.49 ± 26.19^{b}	29.62 ± 3.15
β-carotene bleaching inhibition	422.72 ± 92.91^{b}	497.34 ± 107.67^{b}	661.11 ± 21.93^{a}	2.63 ± 0.14
TBARS inhibition	511.01 ± 17.28^{a}	508.44 ± 4.43^{a}	183.48 ± 3.52^{b}	3.73 ± 1.90
Antitumor activity (GI_{50} values, µg/mL)	Infusion	Decoction	Plant methanol extract	Positive control**
MCF-7 (breast carcinoma)	>400	>400	>400	0.91±0.04
NCI-H460 (non-small lung cancer)	>400	>400	>400	1.42±0.00
HCT-15 (colon carcinoma)	298.23±11.58^{a}	>400	250.24±18.38^{b}	1.91±0.06
HeLa (cervical carcinoma)	277.67±9.04^{a}	>400	259.36±7.57^{b}	1.14±0.21
HepG2 (hepatocellular carcinoma)	>400	>400	>400	3.22±0.67
Hepatotoxicity (GI_{50} value, µg/mL)				
PLP2	>400	>400	>400	2.06±0.03

*Results reported in Barros et al., 2010. **Trolox and ellipticine for antioxidant and antitumor activity assays, respectively. EC_{50} values correspond to the sample concentration achieving 50% of antioxidant activity or 0.5 of absorbance in reducing power assay. GI_{50} values correspond to the sample concentration achieving 50% of growth inhibition in human tumor cell lines or in liver primary culture PLP2. In each row different letters mean significant differences ($p<0.05$).

The infusion and decoction lyophilized samples gave similar antioxidant activity properties and were better than the plant methanol extract in the radical scavenging assays (DPPH and β-carotene bleaching inhibition). Otherwise, the plant methanol extract gave

higher reducing power and lipid peroxidation inhibition measured by TBARS assay (lowest EC_{50} values). The mechanisms involved in the assays used to evaluate antioxidant activity are different and, therefore, each plant preparation can have different compounds with specific capacities to participate in those mechanisms.

Trolox and ellipticine were used as positive controls of antioxidant and antitumor activities evaluation assays, but should not be considered as standards and the comparison with extracts/oral preparations results should be avoided, because they are individual compounds and not mixtures.

Regarding antitumor effects, *M. recutita* infusion and plant methanol extract showed to be selective for HCT-15 and HeLa, since no activity was observed against the other cell lines: MCF-7, NCI-H460 and HepG2. Nevertheless, none of the *M. recutita* preparations showed hepatotoxicity in the porcine liver primary cell culture (non-tumor cells; PLP2) (**Table 8**). The plant methanol extract was slightly more potent than the infusion sample in HCT-15 and HeLa human cell lines. Decoction preparation had no antitumor effects at the maximal concentration used (400 µg/mL). The results obtained are in agreement with other authors that reported minimal growth inhibitory effects in normal cells, but a significant reduction in cell viability in various human cancer cell lines, mainly from methanolic fractions rather than aqueous ones (Srivastava & Gupta, 2007, 2009). The absence of antitumor activity in decoction of another chamomile species, *C. nobile* (Roman chamomile), was previously reported by us (Guimarães et al., 2013).

It should be highlighted that *M. recutita* has been included in commercial mixtures for different pharmacological applications such as i) TBS-101 (a mixture of seven standardized botanical extracts) that showed an outstanding safety profile with significant anticancer activity against androgen-refractory human prostate cancer PC-3 cells, both *in vitro* and *in vivo* (Evans et al., 2009); ii) STW 5 (a mixture of nine standardized botanical extracts) for treatment of gastrointestinal disorders, with a mechanism of action related to their antioxidant properties (Schempp et al., 2006). Nevertheless, chemical characterization and bioactivity evaluation of *M. recutita* infusion and decoction, the most consumed preparations of this herb, have been discarded being addressed herein.

3.2.2. Organic acids and phenolic compounds

Oxalic, quinic, malic, citric and succinic acids were quantified in all the extracts of *M. recutita*, oxalic acid being the most abundant organic acid (**Table 9**).

Table 9. Identification and quantification of organic acids in wild *Matricaria recutita* (mean ± SD).

Organic acid	Infusion	Decoction	Plant (control)
Oxalic acid	8.45 ± 0.32[a]	8.60 ± 0.47[a]	3.24 ± 0.05b
Quinic acid	0.24 ± 0.00b	0.88 ± 0.19[a]	0.17 ± 0.00c
Malic acid	2.26 ± 0.06[a]	1.97 ± 0.03b	0.39 ± 0.02c
Shikimic acid	0.02 ± 0.00	0.02 ± 0.00	tr
Ascorbic acid	nd	nd	tr
Citric acid	6.44 ± 0.85[a]	6.14 ± 0.14[a]	1.55 ± 0.00[b]
Succinic acid	7.00 ± 0.21[a]	5.74 ± 0.13b	1.94 ± 0.05[c]
Fumaric acid	0.01 ± 0.00	0.01 ± 0.00	tr
Total (g/100 g dw)	24.42 ± 1.32[a]	23.35 ±0.65[a]	7.30 ± 0.03[b]

In each row different letters mean significant differences ($p<0.05$); tr- traces; nd-not detected; dw- dry weight.

Shikimic and fumaric acids were present in low or vestigial amounts. Ascorbic acid was not detected neither in infusion nor decoction, and was present in traces in the plant extract. Infusion and decoction gave similar contents in organic acids, and higher than the plant extract.

Organic acids might have antioxidant properties, such is the case of citric and oxalic acids (Hraš et al., 2000; Kayashima & Katayama, 2002), which may contribute for the antioxidant activity of the samples studied herein.

The main phenolic compounds found in *M. recutita* plant and in its decoction and infusion were phenolic acids and derivatives, as also flavonoids such as flavonols and flavones (**Tables 10** and **11**). The chromatographic profile of the three plant preparations can be observed in **Figure 11**. Up to twenty phenolic compounds, including phenolic acids and flavonoids were detected in the *M. recutita* preparations (**Table 10**).

Table 10. Retention time (Rt), wavelengths of maximum absorption in the UV-vis region (λ_{max}), pseudomolecular and MS^2 fragment ions (in brackets, relative abundances) and tentative identification of the phenolic compounds of wild *Matricaria recutita.*

Peak	Rt (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification
1	7.69	328	353	191(100), 179(53), 173(5), 161(5), 135(48)	3-*O*-caffeolyquinic acid
2	8.24	294	153	109(100)	Protocatechuic acid
3	9.75	320	515	515(100),353(5), 191(33), 179(6), 161(13)	1,5-Dicaffeolyquinic acid
4	10.84	326	353	191(75), 179(84), 173(100), 161(11), 135(77)	4-*O*-Caffeolyquinic acid
5	11.34	326	353	191(100), 179(7), 173(3), 161(4), 135(2)	*cis*-5-*O*-Caffeolyquinic acid
6	11.57	326	353	191(100), 179(15), 173(8), 161(15), 135(7)	*trans*-5-*O*-Caffeolyquinic acid
7	12.24	312	355	193(100), 179(13), 149(80)	*cis*-Ferulic acid hexoside
8	12.39	312	355	193(100), 179(13), 149(81)	*trans*-Ferulic acid hexoside
9	14.85	328	367	193(16), 191(100), 173(19), 134(8)	5-*O*-Feruloylquinic acid
10	15.18	354	479	317(100)	Myricetin *O*-hexoside
11	15.45	322	711	549(3), 355(36), 193(100), 149(84)	Ferulic acid hexoside dímer
12	15.81	322	677	515(100), 497(2), 353(17), 335(7), 191(4), 179 (7), 173 (1), 135 (3)	1,3,5-*O* or 1,4,5-*O*-Tricaffeolyquinic
13	16.54	340	651	489(96), 447(55), 285(45)	Luteolin acetylhexoside hexoside
14	17.11	330	515	515 (100),353(66), 335(25),299(2),255(3),203(3),191(26),179(53),173(68), 161(9)	3,4-*O*-Dicaffeolyquinic acid
15	17.68	340	447	285(100)	Luteolin-7-*O*-glucoside
16	18.09	330	515	353(100),335(8),191(89),179(75),173(11),161(7), 155(2),135(28)	3,5-*O*-Dicaffeolyquinic acid
17	18.54	372	505	343(8), 301(100)	Quercetin 7-*O*-acetylhexoside
18	18.64	343	489	327(5), 285(64)	Luteolin *O*-acylhexoside
19	18.81	328	515	353(100),335(4),299(5),255(5),203(3),191(30),179(67), 173(95), 161(2),155(3),135(24)	4,5-*O*-Dicaffeoylquinic acid
20	19.10	296(sh),336	489	327(5), 285(64)	Luteolin *O*-acylhexoside

Compound 2 was identified as protocatechuic acid by comparison of its UV spectrum and retention time with a commercial standard. Thirteen hydroxycinnamic acid derivatives (peaks 1, 3-9, 11, 12, 14, 16 and 19) were detected, ten being quinic acid derivatives (1, 3-6; 9, 12, 14, 16 and 19), whose identities were assigned based on their MS spectra and fragmentation patterns. These compounds released characteristic MS^2 fragment ions at *m/z* 191 (deprotonated quinic acid), 179 (deprotonated caffeic acid) or 193 (deprotonated ferulic acid), which together with their pseudo molecular ions $[M-H]^-$ at *m/z* 353, 515, 677 or 367 allowed their identification as quinic acid derivatives containing one, two or three caffeic acid moieties or one ferulic acid moiety, respectively. The assignments of the different caffeoylquinic acid isomers were made using the recommended IUPAC numbering system, as also the hierarchical keys previously developed by Clifford, 2003 and Clifford et al., 2005.

Compound 6 was positively identified as 5-*O*-caffeoylquinic acid by comparison with an authentic standard, as also to its MS fragmentation pattern. Compound 5 was tentatively assigned as the corresponding *cis* isomer of 5-*O*-caffeoylquinic acid based on its fragmentation pattern and lower levels compared with peak 6. Furthermore, hydroxycinnamoyl *cis* derivatives would be expected to elute before the corresponding *trans* ones, as observed after UV irradiation (366 nm, 24 h) of hydroxycinnamic acids in our laboratory.

Compound 1 ($[M-H]^-$ at *m/z* 353) was identified as 3-*O*-caffeoylquinic acid, yielding the base peak at *m/z* 191 and the ion at *m/z* 179 with an intensity >55% base peak, characteristic of 3-acylchlorogenic acids as reported by Clifford et al. (2003, 2005). Compound 4 was easily distinguished from the other two isomers by its base peak at *m/z* 173 [quinic acid-H-H_2O]$^-$, accompanied by a secondary fragment ion at *m/z* 179 with approximately 84% abundance of base peak, which allowed identifying it as 4-*O*-caffeoylquinic acid according to the fragmentation pattern described by Clifford et al. (2003, 2005). Compound 9 was tentatively identified as 5-*O*-feruloylquinic acid taking into account its fragmentation pattern and relative intensities similar to 5-*O*-caffeoylquinic acid.

Compounds 3, 14, 16 and 19 ($[M-H]^-$ at *m/z* 515) could be assigned as dicaffeoylquinic acids and were assigned to 1,5-*O*-, 3,4-*O*-, 3,5-*O*- and 4,5-*O*- dicaffeoylquinic acids, respectively, based on their elution order, fragmentation pattern and relative abundances (Clifford et al., 2003, 2005). MS^2 fragmentation of compound 14 yielded the formation of signals corresponding to "dehydrated" fragment ions at *m/z* 335 [caffeoylquinic acid –

H-H_2O]$^-$ and *m/z* 173 [quinic acid-H-H_2O]$^-$, characteristic of 4-acyl-caffeoylquinic acids. Furthermore, according to Clifford et al., 2005, the intensity of signal at *m/z* 335 (25% of base peak) is more intense than in 4,5-*O*-dicaffeoylquinic (barely detectable, 4% of base peak). These observations allowed assigning compound 14 as 3,4-*O*-dicaffeoylquinic acid. The fragmentation pattern for 3,5-*O*-dicaffeoylquinic (compound 16) acid was similar to the one previously reported by Clifford et al., 2005. MS^2 base peak was at *m/z* 353, produced by the loss of one of the caffeoyl moieties [M-H-caffeoyl]$^-$, and subsequent fragmentation of this ion yielded the same fragments as 5-caffeoylquinic acid at *m/z* 191, 179 and 135, although in this case with a comparatively more intense signal at *m/z* 179 [caffeic acid-H]$^-$ (75% base peak). Compound 19 was assigned to 4,5-*O*- dicaffeoylquinic acid since its fragmentation was identical to those previously reported by Clifford et al., 2005. In this case, the signal at *m/z* 335 was barely detectable (<5 % of base peak) and the intense signal at *m/z* 173, is characteristic for an isomer substituted at position 4, which indicated that whereas 3,4-*O*-dicaffeoylquinic acid initially loses the caffeoyl moiety at position 3, in the case of 4,5-*O*-dicaffeoylquinic acid would initially lose that at position 5. Compound 3 was assigned as 1,5-*O*-dicaffeoylquinic acid, following the criteria the reported by Clifford et al., 2005, the weak ions at *m/z* 335 and 179 (<10 % of base peak). Compound 12 was identified as tricaffeoylquinic acid according to its pseudomolecular ion [M-H]$^-$ at *m/z* 677 and diagnostic MS^2 fragments at *m/z* 515 (loss of the first caffeoyl), *m/z* 353 (loss of the second caffeoyl), *m/z* 191 (loss of the third caffeoyl to give quinic acid ion), *m/z* 179 (caffeic acid ion). The signal observed at *m/z* 497 can be interpreted by the loss of a water molecule from the ion at *m/z* 515. According to the relative intensities of different tricaffeoylquinic acid isomers reported by Lin & Harnly (2008), this compound could be assigned as 1,3,5-*O*-tricaffeoylquinic acid or 1,4,5-*O*-tricaffeoylquinic acid.

Compounds 7 and 8 with MS^2 fragments at *m/z* 193 ([ferulic acid-H]$^-$) resulting from the loss of a hexosyl moiety, -162 mu, and 176 ([ferulic acid-H-H_2O]$^-$) was tentatively assigned as a ferulic acid hexoside. Compound 7 presented similar UV and mass spectra characteristics as peak 8 but an earlier retention time, taking into account the observation above it was tentatively identified as *cis* ferulic acid hexoside. Similarly, compound 11 was tentatively identified as ferulic acid hexoside dimer.

Flavones were the most abundant flavonoids present in the samples analysed (**Tables 11** and **12**).

Table 11. Quantification of phenolic compounds in wild *Matricaria recutita* (mean ± SD).

Peak	Infusion	Decoction	Plant (control)
3-*O*-caffeolyquinic acid	0.15 ± 0.01[a]	0.12 ± 0.00[b]	0.07 ± 0.00[c]
Protocatechuic acid	0.07 ± 0.01[a]	0.05 ± 0.01[b]	nd
1,5-Dicaffeolyquinic acid	nd	tr	0.02 ± 0.00
4-*O*-Caffeolyquinic acid	0.21 ± 0.00[b]	0.13 ± 0.00[c]	0.24 ± 0.00[a]
cis 5-*O*-Caffeolyquinic acid	0.17 ± 0.04[a]	0.15 ± 0.00[a]	nd
trans 5-*O*-Caffeolyquinic acid	0.26 ± 0.04[b]	0.22 ± 0.01[b]	1.02 ± 0.02[a]
cis Feruloyl hexoside acid	0.32 ± 0.02[a]	0.28 ± 0.01[b]	nd
trans Feruloyl hexoside acid	0.46 ± 0.01[b]	0.38 ± 0.01[c]	1.02 ± 0.00[a]
5-*O*-Feruloylquinic acid	0.03 ± 0.00[a]	0.02 ± 0.00[b]	nd
Myricetin *O*-hexoside	nd	nd	0.05 ± 0.00
Feruloyl hexoside acid dímer	0.59 ± 0.00[b]	0.55 ± 0.01[c]	0.91 ± 0.00[a]
1,3,5-*O* or 1,4,5-Tricaffeolyquinic	0.03 ± 0.00[b]	0.02 ± 0.00[c]	0.06 ± 0.00[a]
Luteolin acetylhexoside hexoside	0.02 ± 0.00[c]	0.03 ± 0.00[b]	0.11 ± 0.01[a]
3,4-*O*-Dicaffeolyquinic acid	0.73 ± 0.03[a]	0.33 ± 0.01[b]	0.35 ± 0.00[b]
Luteolin-7-*O*-glucoside	0.17 ± 0.01[a]	0.09 ± 0.00[b]	0.06 ± 0.00[c]
3,5-*O*-Dicaffeolyquinic acid	0.26 ± 0.07[a]	0.16 ± 0.00[b]	0.10 ± 0.00[b]
Quercetin 7-*O*-acetylhexoside	nd	nd	0.10 ± 0.00
Luteolin *O*-acylhexoside	0.09 ± 0.01[b]	0.06 ± 0.00[c]	0.19 ± 0.01[a]
4,5-*O*-Dicaffeoylquinic acid	0.17 ± 0.03[a]	0.13 ± 0.01[b]	nd
Luteolin *O*-acylhexoside	1.29 ± 0.12[b]	0.81 ± 0.04[c]	2.10 ± 0.00[a]
Total phenolic acids (g/100 g dw)	3.43 ± 0.22[b]	2.53 ± 0.02[c]	3.99 ± 0.02[a]
Total flavonoids (g/100 g dw)	1.56 ± 0.12[b]	0.98 ± 0.04[c]	2.59 ± 0.01[a]
Total phenolic compounds (g/100 g dw)	5.00 ± 0.33[b]	3.51 ± 0.06[c]	6.58 ± 0.03[a]

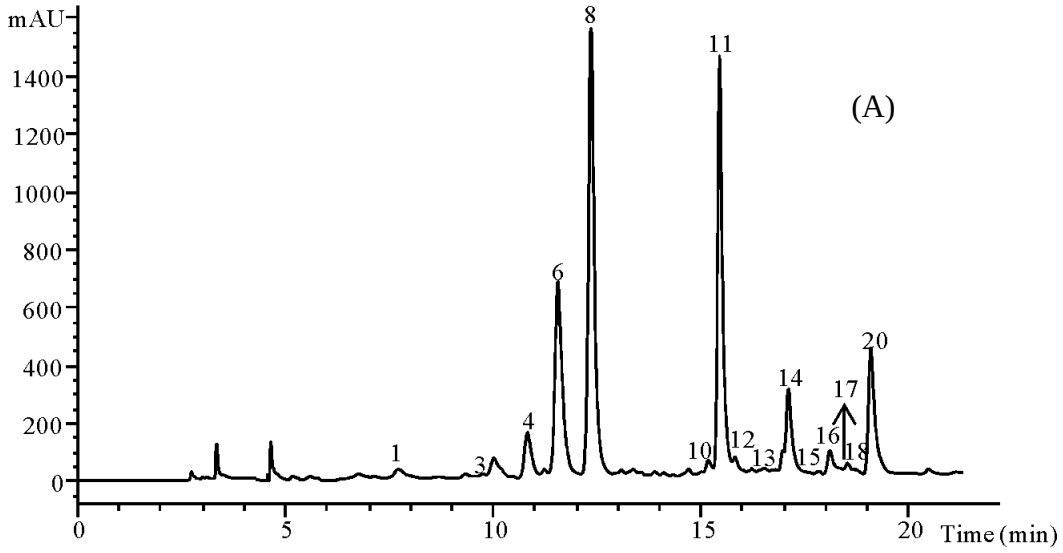
In each row different letters mean significant differences ($p<0.05$); tr- traces; nd-not detected; dw- dry weight.

Compounds 13, 15, 18 and 20 were identified as luteolin derivatives. Compounds 15, 18 and 20 presented a pseudomolecular ions [M-H]- at *m/z* 447 and 489 releasing a common MS^2 fragment at *m/z* 285 ($[M-H-162]^-$ and $[M-H-42-162]^-$, loss of hexosyl and acetylhexoside moieties, respectively). They were tentatively identified as luteolin 7-*O*-glucoside (retention time compared with a commercial standard), and two luteolin *O*-acetylhexoside (compound 18 and 20), respectively. Compound 13 presented a pseudomolecular ion $[M-H]^-$ at *m/z* 651 releasing three MS^2 fragments at *m/z* 489, 447 and 285 (loss of hexosyl, acetylhexosyl and acetyldihexosyl moieties, respectively), being tentatively identified as luteolin acetylhexoside hexoside.

Flavonols (compounds 10 and 17) were also found in the studied samples (**Table 11**). Compound 10 presented a pseudomolecular ion $[M-H]^-$ at *m/z* 479, releasing an MS^2 fragment at *m/z* 317 ($[M-H-162]^-$, loss of an hexosyl moiety), corresponding to myricetin, which allowed a tentative identification of the compound as myricetin *O*-hexoside. Compound 17 corresponded to a quercetin derivative presenting a pseudomolecular ion

$[M-H]^-$ at *m/z* 505 releasing an MS^2 fragment at *m/z* 301 (quercetin; $[M-H-42-162]^-$, loss of an acetylhexoside moiety). It is known that the introduction of a glycoside on the hydroxyls at positions 7, 3´ or 4´ has no effect on wavelength maxima or the spectrum shape in relation to the aglycone (Mabry et al., 1970). Thus, quercetin 7-*O*-glycosides would have λ_{max} in Band I around 370 nm, while quercetin 3 *O*-glycosides are hypsochromically shifted to around 354 nm. Since this compound presented λ_{max} at 372 nm it was tentatively identified as quercetin 7-*O*-acetylhexoside.

The amounts of the phenolic compounds found varied among the different preparations and flavonols decreased in infusions and decoctions, but otherwise *cis* isomer of caffeoylquinic and ferulic acids derivatives appeared in these preparations (**Table 11** and **Figure 11**).



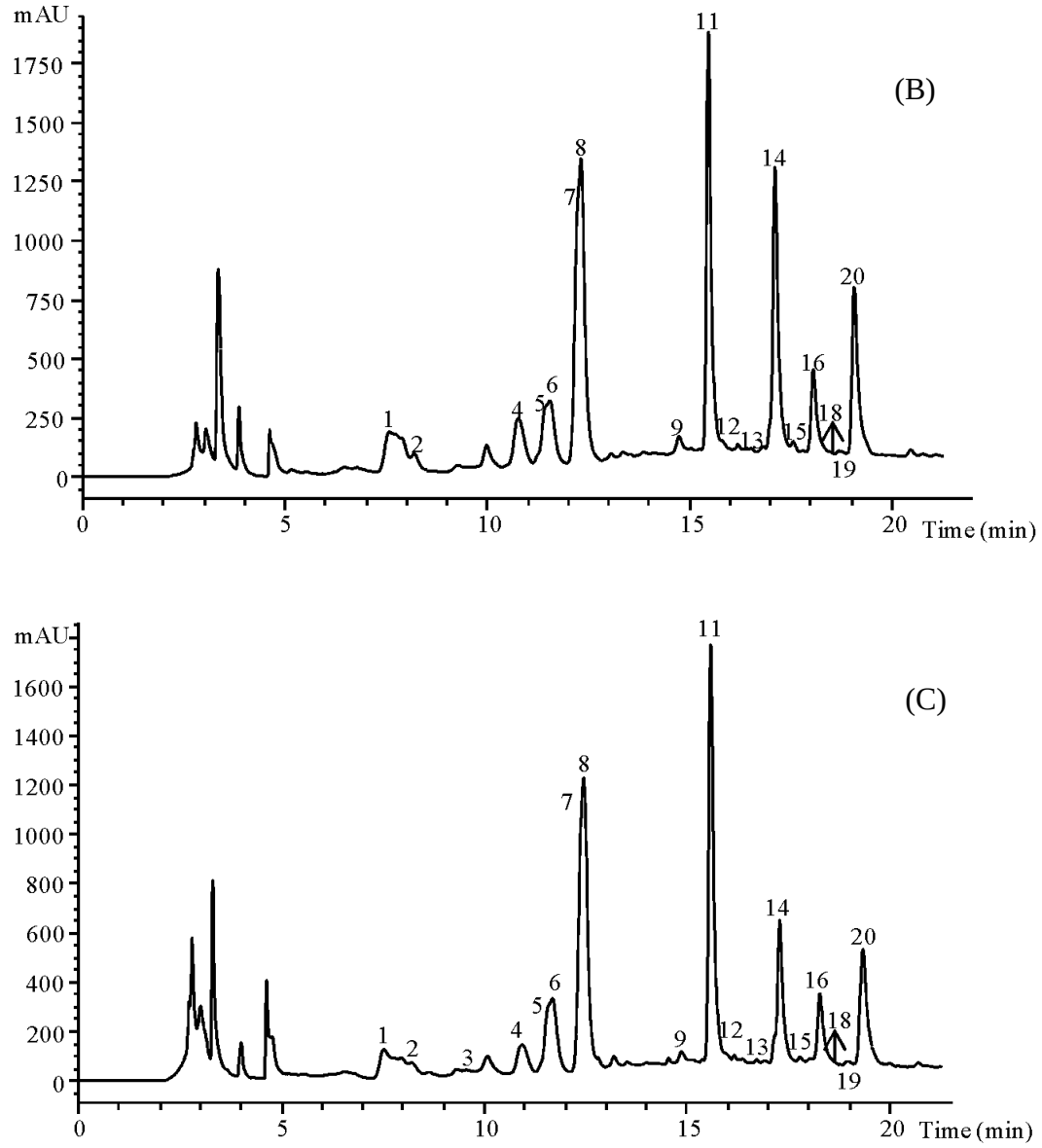


Figure 11. HPLC chromatogram of the phenolic compounds of *Matricaria recutita* recorded at 280 nm (A) plant methanol extract (control; 1:2 v/v); (B) infusion and (C) decoction.

The plant methanol extract (control) presented the highest amounts of phenolic acids (3.99g/100 g dw) and flavonoids (2.59g/100 g dw) as also total phenolic compounds (6.58g/100 g dw), followed by infusion (5.00g/100 g dw) and decoctions (3.51g/100 g dw). The same was observed in *C. nobile* (Roman chamomile) in a previously work of our research group (Guimarães et al., 2013).

The major compound found in the herbal plant and in the preparations was luteolin *O*-acylhexoside (compound 20). Mulinacci et al. (2000), Nováková et al. (2010), Harbourne et al., (2009) and Srivastava & Gupta, (2009) reported the presence of apigenin 7-*O*-glucoside and other apigenin derivatives, but these compounds were not detected in our

samples. Furthermore, Mulinacci et al. (2000) revealed the presence of different flavonoids, such as patuletin and other quercetin derivatives. In relation to the quantification, no comparison can be made, due to the fact that those authors only presented percent area measured at 335 nm of the main phenolic compounds found.

Nováková et al. (2010) presented the phenolic profile of methanolic extracts of *M. recutita* herbal flowers and infusions, demonstrating also lower values in infusions preparation when compared to the herbal plant methanolic extract. These authors also found a dissimilar profile than the one obtained in this study, finding other flavonoids, such as kaempferol, isorhamnetin and different quercetin derivatives in their samples. The quantification was expressed in µmol/L, being difficult to compare with the results of *M. recutita* obtained herein.

Overall, it was observed a decrease in the amount of phenolic compounds in the plant infusion or decoction, compared to the methanol extract; the same was not observed for organic acids, which indicates that these compounds are better extracted with hot water than with methanol. The decoction had no antitumor effects which could indicate that these effects might be related to compounds (including phenolic compounds) that were not extracted or that were affected by the decoction procedure. Both plant methanol extract and infusion showed inhibitory activity of the growth of colon and cervical carcinoma cell lines, without toxicity for hepatocyte normal cells. Therefore, wild German chamomile (*M. recutita*) may be considered a source of important phytochemicals with bioactive properties to be explored in the medicine, food, and cosmetic industries

3.3. *Arbutus unedo, Prunus spinosa, Rosa canina* and *Rosa micrantha* fruits

3.3.1. Phenolic compounds

The characterization of the phenolic compounds present in the wild fruits was performed by HPLC-DAD-MS analysis, and data of the retention time, λ_{max}, pseudomolecular ion, main fragment ions in MS^2, tentative identification and quantification of phenolic acids, flavone/ols, flavan-3-ols, and anthocyanins are presented in **Tables 12-14**.

Table 12. Retention time (Rt), wavelengths of maximum absorption in the UV-vis region (λ_{max}), pseudomolecular and MS^2 fragment ions (in brackets, relative abundances), tentative identification and quantification of the phenolic acids and flavone/ols of wild fruits.

Peak	Rt (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Identification type	Quantification (mg/100g)
Arbutus unedo							
1	18.7	354	615	615(100),463(18),301(18)	Quercetin galloylhexoside derivative	DAD/MS	0.57 ± 0.03
2	18.9	352	615	615(100),463(36),301(29)	Quercetin galloylhexoside derivative	DAD/MS	1.31 ± 0.00
3	19.2	-	463	317(100)	Myricetin rhamnoside	DAD/MS	0.23 ± 0.01
4	19.5	348	609	301(100)	Quercetin 3-*O*-rutinoside	Standard/DAD/MS	1.70 ± 0.02
5	19.9	354	447	285(100)	Kaempferol hexoside	DAD/MS	0.84 ± 0.14
6	20.4	348	463	301(100)	Quercetin 3-*O*-glucoside	Standard/DAD/MS	2.34 ± 0.01
7	20.8	352	463	317(100)	Myricetin rhamnoside	DAD/MS	0.45 ± 0.01
8	23.7	352	433	301(100)	Quercetin pentoside	DAD/MS	1.32 ± 0.04
9	24.7	350	447	301(100)	Quercetin rhamnoside	DAD/MS	2.10 ± 0.04
					Total		10.86 ± 0.24
Prunus spinosa							
1	5.2	326	353	191(100),179(98),161(14),135(75)	3-*O*-caffeoylquinic acid	Clifford et al. 2003 and 2005/ DAD/MS	22.09 ± 0.11
2	7.1	312	337	191(36),163(100),155(8),119(75)	3-*p*-coumaroylquinic acid	Clifford et al. 2003 and 2005/ DAD/MS	0.80 ± 0.01
3	7.5	324	353	191(74),179(94),173(100),161(8),135(75)	4-*O*-caffeoylquinic acid	Clifford et al. 2003 and 2005/ DAD/MS	3.41 ± 0.03
4	8.2	326	367	193(100),191(16),173(14),149(25)	3-*O*-feruloylquinic acid	Clifford et al. 2003 and 2005/ DAD/MS	1.76 ± 0.04
5	8.5	340	401	269(100)	Apigenin pentoside	DAD/MS	1.32 ± 0.03
6	11.1	312	337	191(3),173(100),163(43),155(12),119(21)	4-*p*-coumaroylquinic acid	Clifford et al. 2003 and 2005/ DAD/MS	0.64 ± 0.00
7	14.3	346	739	625(100),607(7),501(9),475(7),317(14),299(24)	Myricetin derivative	DAD/MS	1.06 ± 0.05
8	15.1	328	335	179(20),161(100),135(49)	Caffeoyl hexoside	DAD/MS	1.07 ± 0.00
9	18.9	350	595	301(100)	Quercetin pentosyl-hexoside	DAD/MS	1.36 ± 0.04

Peak	Rt (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Identification type	Quantification (mg/100g)
10	19.5	356	609	301(100)	Quercetin rhamnosyl-hexoside	DAD/MS	2.22 ± 0.03
11	19.8	356	609	301(100)	Quercetin 3-*O*-rutinoside	Standard/DAD/MS	15.63 ± 0.33
12	20.3	352	595	301(100)	Quercetin pentosyl-hexoside	DAD/MS	6.83 ± 0.29
13	20.7	356	463	301(100)	Quercetin 3-*O*-glucoside	Standard/DAD/MS	1.36 ± 0.02
14	21.2	354	463	301(100)	Quercetin hexoside	DAD/MS	4.70 ± 0.50
15	22.9	350	593	285(100)	Kaempferol 3-*O*-rutinoside	Standard/DAD/MS	1.90 ± 0.05
16	23.8	354	609	447(4),301(100)	Quercetin hexosyl-rhamnoside	DAD/MS	7.17 ±0.42
17	24.0	354	433	301(100)	Quercetin pentoside	DAD/MS	8.84 ± 0.26
18	24.4	358	623	315(100)	Isorhamnetin 3-*O*-rutinoside	Standard/DAD/MS	0.87 ± 0.03
19	25.1	348	447	301(100)	Quercetin rhamnoside	DAD/MS	1.30 ± 0.02
20	25.8	356	505	463(5),301(100)	Quercetin acetylhexoside	DAD/MS	1.90 ± 0.05
21	29.8	348	651	609(15),301(100)	Quercetin acetylrutinoside	DAD/MS	1.00 ± 0.01
					Total phenolic acids		29.78±0.17
					Total flavone/ols		57.48±0.74
					Total		87.25 ± 0.91
				Rosa canina			
1	15.9	292	435	303(24),285(100)	Taxifolin pentoside	DAD/MS	1.18 ± 0.03
2	16.9	294	449	287(20),269(100),225(2),209(2),151(27)	Eriodictyol hexoside	DAD/MS	0.15 ± 0.00
3	17.6	290	449	449(100),287(44),269(64),225(14),209(14),151(55)	Eriodictyol hexoside	DAD/MS	0.50 ± 0.01
4	19.6	356	609	301(100)	Quercetin 3-*O*-rutinoside	Standard/DAD/MS	0.47 ± 0.00
5	20.0	352	477	301(100)	Quercetin glucuronide	DAD/MS	0.24 ± 0.01
6	20.4	290	435	303(26),285(100)	Taxifolin pentoside	DAD/MS	0.83 ± 0.00
7	20.5	358	463	301(100)	Quercetin 3-*O*-glucoside	Standard/DAD/MS	0.66 ± 0.01
8	20.9	352	463	301(100)	Quercetin hexoside	DAD/MS	0.78 ± 0.00
9	23.8	352	433	301(100)	Quercetin pentoside	DAD/MS	0.10 ± 0.01
10	24.2	346	623	315(100)	Isorhamnetin 3-*O*-rutinoside	Standard/DAD/MS	0.02 ± 0.01
11	24.6	350	447	301(100)	Quercetin rhamnoside	DAD/MS	0.46 ± 0.01
12	35.4	348	593	285(100)	Kaempferol rhamnosyl-hexoside	DAD/MS	0.09 ± 0.00
					Total		5.50 ± 0.05

Peak	Rt (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Identification type	Quantification (mg/100g)
Rosa micrantha							
1	5.7	278	345	183(100),168(10)	Methyl gallate hexoside	DAD/MS	2.45 ± 0.02
2	16.0	294	435	303(54),285(100)	Taxifolin pentoside	DAD/MS	2.68 ± 0.01
3	17.1	296	449	449(100), 287(10),269(85),225(12),209(12),151(7)	Eriodictyol hexoside	DAD/MS	0.22 ± 0.01
4	17.8	290	449	449(100),287(44),269(66),225(12),209(25),151(44)	Eriodictyol hexoside	DAD/MS	0.63 ± 0.02
5	19.8	354	609	301(100)	Quercetin 3-*O*-rutinoside	Standard/DAD/MS	0.32 ± 0.00
6	20.3	358	477	301(100)	Quercetin glucuronide	DAD/MS	0.10 ± 0.01
7	20.6	292	435	303(53),285(100)	Taxifolin pentoside	DAD/MS	1.92 ± 0.00
8	20.7	352	463	301(100)	Quercetin 3-*O*-glucoside	Standard/DAD/MS	0.97 ± 0.01
9	21.2	352	463	301(100)	Quercetin hexoside	DAD/MS	0.90 ± 0.01
10	24.1	352	433	301(100)	Quercetin pentoside	DAD/MS	0.21 ± 0.00
11	24.4	352	623	315(100)	Isorhamnetin 3-*O*-rutinoside	Standard/DAD/MS	tr
12	25.1	346	447	301(100)	Quercetin rhamnoside	DAD/MS	0.71 ± 0.01
13	35.7	348	593	285(100)	Kaempferol rhamnosyl-hexoside	DAD/MS	0.05 ± 0.00
					Total		11.16 ± 0.04

tr-traces

Table 13. Retention time (Rt), wavelengths of maximum absorption in the UV-vis region (λ_{max}), pseudomolecular and MS^2 fragment ions (in brackets, relative abundances), tentative identification and quantification of flavan-3-ols and galloyl derivatives in wild fruits.

Peak	Rt (min)	λ_{max} (nm)	Molecular ion [M-H] (*m/z*)	MS^2 (*m/z*)	Tentative identification	Identification type	Quantification (mg/100g)
				Arbutus unedo			
1	9.9	274	343	191 (100),169 (13)	Galloylquinic acid	Pawlowska et al., 2006/ Mendes et al., 2011 and Tavares et al., 2010/DAD/MS	8.65 ± 0.00
2	11.3	278	331	169 (100)	Galloylhexoside acid	DAD/MS	1.02 ± 0.03
3	12.5	276	593	423(75),407(25),305 (100)	PA dimer (GC+C)[b]	DAD/MS	1.07 ± 0.00
4	12.9	276	325	169 (100)	Galloyl shikimic acid	Pawlowska et al., 2006/ Mendes et al., 2011 and Tavares et al., 2010/DAD/MS	3.61 ± 0.01
5	15.7	274	577	451(17),425(26),407(100), 289(78), 287(7)	B1 dimer	DAD/MS	8.56 ± 0.31
6	16.4	280	865	865(100),739(9), 713(9), 695(17), 577(9), 575(9), 425(12), 407(17), 289(12),287(12)	B-type procyanidin trimer	DAD/MS	4.11 ± 0.14
7	17.5	278	865	865(100),739(3), 713(3), 695(2), 577(11), 575(3), 425(4), 407(5), 289(9),287(7)	B-type procyanidin trimer	DAD/MS	2.76 ± 0.07
8	18.3	278	289	245 (70), 205 (33), 151 (21), 137 (33)	(+)-catechin	Standard/ DAD/MS	13.51 ± 0.93
9	18.4	278	495	343(42),191(100)	Digalloylquinic acid	Pawlowska et al., 2006/ Mendes et al., 2011 and Tavares et al., 2010/DAD/MS	3.49 ± 0.42
10	19.4	278	1153	865 (7), 577 (8), 289 (100)	B-type procyanidin tetramer	DAD/MS	2.94 ± 0.07
11	22.4	278	577	451(17),425(75),407(100), 289(58), 287(17)	B-type procyanidin dimer	DAD/MS	3.35 ± 0.06
12	22.9	276	477	325(100),169(29)	Digalloylquinic shikimic acid	Pawlowska et al., 2006/ Mendes et al., 2011 and Tavares et al., 2010/DAD/MS	1.81 ± 0.04
13	23.9	270	633	633(100),463(12),301(70),275(5)	Strictinin ellagitannin	Pawlowska et al., 2006/ Mendes et al., 2011 and Tavares et al., 2010/DAD/MS	3.54 ± 0.01
14	28.3	270	477	325(100),169(54)	Digalloylquinic shikimic acid	Pawlowska et al., 2006/ Mendes et al., 2011 and Tavares et al., 2010/DAD/MS	2.51 ± 0.00
					Total flavan-3-ols		36.30 ± 0.70
					Total galloyl derivatives		24.63 ± 0.42
					Total		60.93 ± 0.27

Peak	Rt (min)	λ_{max} (nm)	Molecular ion [M-H] (*m/z*)	MS^2 (*m/z*)	Tentative identification	Identification type	Quantification (mg/100g)
				Rosa canina			
1	11.5	280	1351	1019(9),899(26),675(100),451(96),287(9)	PA trimer triglycoside	DAD/MS	0.77 ± 0.00
2	12.3	280	1189	1189(100), 898(23),739(17),575(10),449(17),289(7)	PA trimer diglycoside	DAD/MS	1.37 ± 0.00
3	12.9	280	819[a]	739(100),674(2),289(15)	PA tetramer triglycoside	Salminen et al., 2005/DAD/MS	0.69 ± 0.04
4	13.5	274	819[a]	739(38),674(2),289(100)	PA tetramer triglycoside	Salminen et al., 2005/DAD/MS	1.21 ± 0.07
5	14.9	280	739	449(18),425(29),407(100),289(47)	PA dimer monoglycoside	DAD/MS	3.27 ± 0.10
6	15.6	280	451	289(100)	(+)-catechin-hexoside	DAD/MS	1.62 ± 0.06
7	15.9	272	577	451(4),425(52),407(100),289(69)	Procyanidin dimer B1	DAD/MS	1.68 ± 0.09
8	16.3	278	577	425(31), 407(100), 289(25)	Procyanidin dimer B3	DAD/MS	1.50 ± 0.13
9	17.7	282	865	865(100),695(10), 577(50), 449(15), 407(25), 287(35)	B-type procyanidin trimer	DAD/MS	0.74 ± 0.08
10	17.9	280	739	739(100),449(11),425(11),407(48),289(22)	Procyanidin dimer monoglycoside	DAD/MS	1.32 ± 0.02
11	18.7	278	289	289(100),245(29), 205(8), 179(7), 137(4)	(+)-catechin	Standard/ DAD/MS	3.59 ± 0.17
12	30.1	280	865	865(100),739(14),577(21),575(21),425(14),407(14),289(21)	B-type procyanidin trimer	DAD/MS	2.14 ± 0.08
					Total flavan-3-ols		19.90 ± 0.51
				Rosa micrantha			
1	11.3	280	1351	1019(23),899(26),675(100),451(73),287(10)	PA trimer triglycoside	DAD/MS	2.32 ± 0.05
2	12.1	280	1189	898(37),739(19),575(14),449(22),289(100)	PA trimer diglycoside	DAD/MS	2.02 ± 0.04
3	12.7	280	819[a]	819(100),739(18),674(4),289(35)	PA tetramer triglycoside	Salminen et al., 2005/DAD/MS	0.88 ± 0.07
4	13.2	280	819[a]	739(18),674(27),289(100)	PA tetramer triglycoside	Salminen et al., 2005/DAD/MS	2.52 ± 0.06
5	13.8	280	1351	1019(13),899(13),675(50),451(100),287(14)	PA trimer triglycoside	DAD/MS	0.49 ± 0.01
6	14.5	280	739	739(100),449(27),425(13),407(63),289(43)	PA dimer monoglycoside	DAD/MS	4.93 ± 0.06
7	15.3	280	451	289(100)	(+)-catechin-hexoside	DAD/MS	2.64 ± 0.14
8	15.6	274	577	451(15),425(55),407(100),289(91)	Procyanidin dimer B1	DAD/MS	1.69 ± 0.04
9	17.6	280	739	739(100),449(25),425(15),407(47),289(22)	Procyanidin dimer monoglycoside	DAD/MS	0.94 ± 0.08
10	18.4	278	289	289(100),245(72), 205(32), 179(16), 137(18)	(+)-catechin	Standard/ DAD/MS	2.90 ± 0.06
11	19.8	284	819[a]	819(100),739(33),674(11),289(67)	PA tetramer triglycoside	Salminen et al., 2005/DAD/MS	1.54 ± 0.07
12	19.9	280	865	865(100),739(15),577(3),575(5),425(2),407(5),289(9)	B-type procyanidin trimer	DAD/MS	0.68 ± 0.12
13	21.6	280	851	851(100),739(6), 671(4), 561(62), 537(17), 407(10), 381(33), 357(6), 289(12)	PA trimer (2C+A)[b]	DAD/MS	1.80 ± 0.06

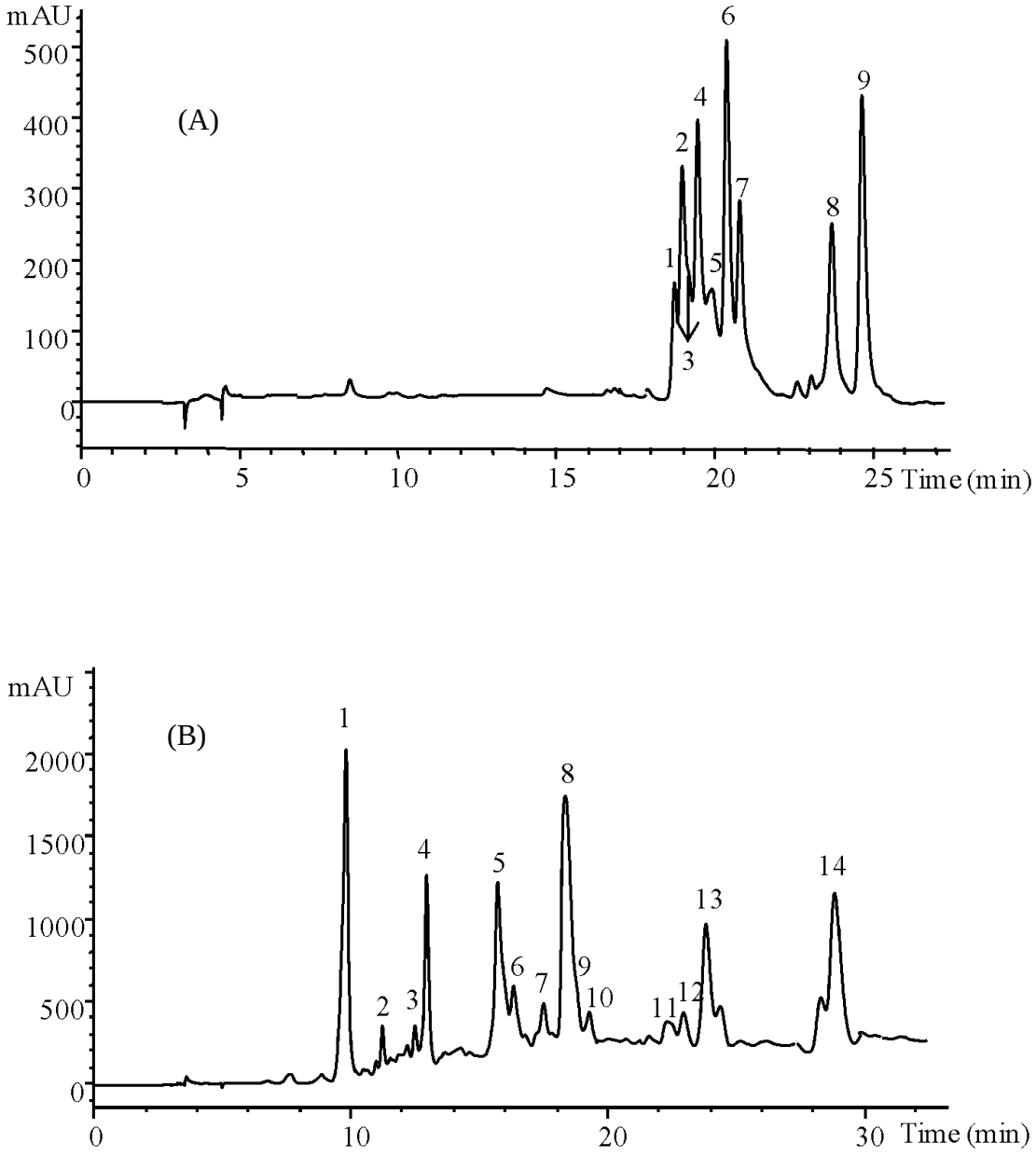
Peak	Rt (min)	λ_{max} (nm)	Molecular ion [M-H] (*m/z*)	MS^2 (*m/z*)	Tentative identification	Identification type	Quantification (mg/100g)
14	25.9	282	739	739(100),449(11),425(33),407(11),289(33)	PA dimer monoglycoside	DAD/MS	1.98 ± 0.11
15	26.8	280	851	851(100),739(6), 671(6), 561(71), 537(18),407(12),381(65), 357(12), 289(12)	PA trimer (2C+A)[b]	DAD/MS	1.40 ± 0.08
16	28.8	280	1027	1027(100),900(4),737(3),575(4),407(15),287(7)	PA trimer monoglycoside	DAD/MS	0.96 ± 0.34
17	29.9	282	865	865(100),739(6),577(25),575(31),425(13),407(25),289(25)	B-type procyanidin trimer	DAD/MS	1.63 ± 0.03
18	35.6	276	577	451(14),425(51),407(71),289(100)	B-type procyanidin dimer	DAD/MS	1.31 ± 0.03
					Total flavan-3-ols		32.62 ± 0.70

[a] $[M-H]^{2-}$; [b] C: (epi)catechin, A: (epi)afzelechin, GC: (epi)gallocatechin

Table 14. Retention time (Rt), wavelengths of maximum absorption in the UV-vis region (λ_{max}), pseudomolecular and MS^2 fragment ions (in brackets, relative abundances), tentative identification and quantification of the anthocyanins of wild fruits.

Peak	Rt (min)	λ_{max} (nm)	Molecular ion $[M+H]^+$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Identification type	Quantification (µg/100g dw)
					Arbutus unedo		
1	18.0	518	465	303(100)	Delphinidin 3-*O*-glucoside	Standard/DAD/MS	0.91 ± 0.01
2	22.4	518	449	287(100)	Cyanidin 3-*O*-glucoside	Standard/DAD/MS	11.40 ± 0.03
3	25.9	518	419	287(100)	Cyanidin 3-*O*-pentoside	Standard/DAD/MS	1.45 ± 0.02
					Total		13.77 ± 0.01
					Prunus spinosa		
1	23.1	518	449	287(100)	Cyanidin 3-*O*-glucoside	Standard/DAD/MS	19.83 ± 0.05
2	24.4	518	595	449(6), 287(100)	Cyanidin 3-*O*-rutinoside	Standard/DAD/MS	31.12 ± 0.11
3	30.9	518	463	301(100)	Peonidin 3-*O*-glucoside	Standard/DAD/MS	10.73 ± 0.16
4	31.4	520	609	463(3), 301(100)	Peonidin 3-*O*-rutinoside	Standard/DAD/MS	34.47 ± 0.03
5	32.2	518	419	287(100)	Cyanidin 3-*O*-pentoside	Standard/DAD/MS	1.49 ± 0.12
6	37.4	520	433	301(100)	Peonidin 3-*O*-pentoside	Standard/DAD/MS	0.26 ± 0.03
7	38.2	520	491	287(100)	Cyanidin 3-*O*-acetylglucoside	DAD/MS	1.77 ± 0.01
8	42.3	520	505	301(100)	Peonidin 3-*O*-acetylglucoside	DAD/MS	0.73 ± 0.05
					Total		100.40 ± 0.47
					Rosa canina		
1	22.6	516	449	287(100)	Cyanidin 3-*O*-glucoside	Standard/DAD/MS	0.68 ± 0.01
					Rosa micrantha		
1	22.5	516	449	287(100)	Cyanidin 3-*O*-glucoside	Standard/DAD/MS	1.19 ± 0.03

As an example, the HPLC profiles of flavonols (A), flavan-3-ols (B) and anthocyanins (C) of *A. unedo* fruits, recorded at 370, 280 and 520 nm, respectively, can be observed in **Figure 12**.



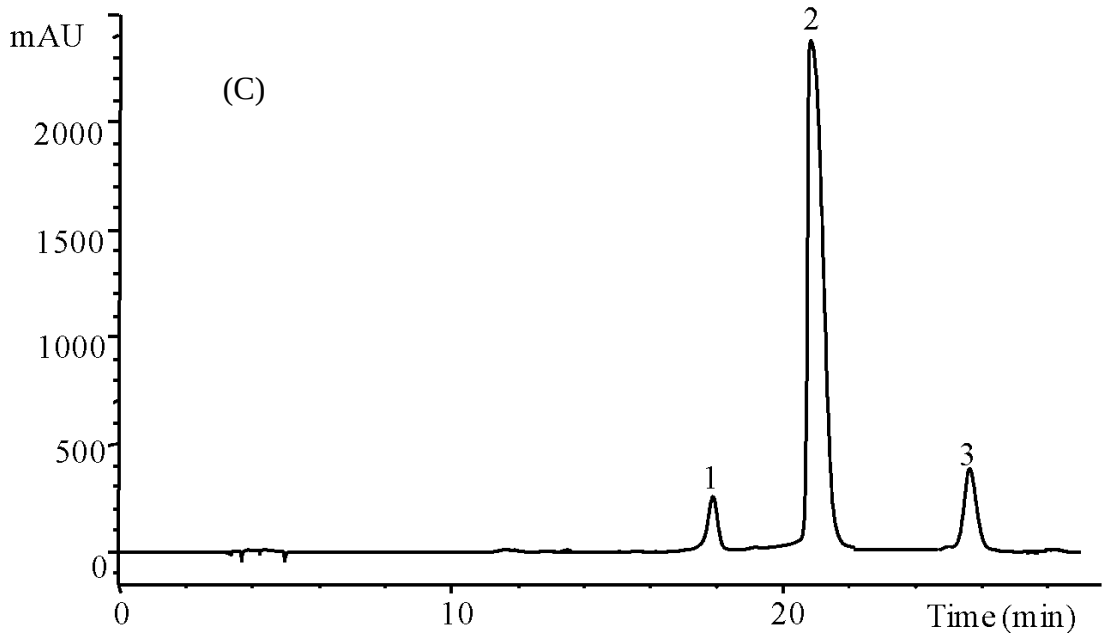


Figure 12. HPLC phenolic profiles of *Arbutus unedo* obtained at 370 nm (A), 280 nm (B) and 520 nm (C) used for recording flavone/ols, flavan-3-ols and galloyl derivatives, and anthocyanins, respectively. Note that different chromatographic conditions were used in each case.

3.3.1.1. *Phenolic acids and flavone/ols*

Prunus spinosa fruits were the only ones that presented phenolic acids, all belonging to the hydroxycinnamic acid derivative sub-group, among them, five compounds (peaks 1-4 and 6) were quinic acid derivatives identified according to their UV spectra (λ_{max} at 314-330 nm) and pseudo molecular ions $[M-H]^-$ (*m/z* at 337, 353 and 367, all of them yielding a product ion at *m/z* 191, due to the deprotonated quinic acid). Peak assignments of the different hydroxycinnamoylquinic acid isomers were made using the recommended IUPAC numbering system (IUPAC, 1976) and the hierarchical keys previously developed by Clifford et al., (2003) and Clifford et al., (2005). Peak 1, the major phenolic compound found in *P. spinosa* fruits, and peak 2 were identified as 3-*O*-caffeoylquinic acid and 3-*p*-coumaroylquinic acid, respectively, as peak 1 yielded deprotonated quinic acid (*m/z* at 191) as base peak and another majority ion corresponding the hydroxycinnamic acid residue at *m/z* 179 [caffeic acid-H]$^-$, and peak 2 presented *m/z* 163 [*p*-coumaric acid-H]$^-$ as base peak, a fragmentation pattern characteristic of 3-acylchlorogenic acids (Clifford et al., 2003, 2005). Similarly, peak 4, with a major ion at *m/z* 193 [*p*-ferulic acid-H]$^-$, was tentatively identified as 3-*O*-feruloylquinic acid taking into account its pseudomolecular ion and fragmentation pattern. Peaks 3 and 6 were easily distinguished from its base peak at *m/z* 173 ([quinic acid-H-H_2O]$^-$), in the case of peak 3,

accompanied by a secondary fragment ion at *m/z* 179 with approximately 94% abundance, which allowed their identification as 4-*O*-caffeoylquinic and 4-*p*-coumaroylquinic acids according to the fragmentation patterns described by Clifford et al. (2003, 2005). Peak 8 presented a UV spectra similar to caffeic acid, with λ_{max} around 328 nm, and a pseudo molecular ion $[M-H]^-$ at *m/z* 335, releasing a fragment at *m/z* 179 [caffeic acid-H]$^-$ (-162 mu, loss of a hexosyl moiety); it was tentatively identified as a caffeoyl hexoside. In all the studied samples, quercetin derivatives (λ_{max} around 354 nm, and an MS^2 fragment at *m/z* 301) were particularly abundant (**Table 13**). Quercetin 3-*O*-rutinoside (peaks: 4- *A. unedo*, 11- *P. spinosa*, 4- *R. canina* and 5- *R. micrantha* in **Table 13**) and quercetin 3-*O*-glucoside (peaks: 6- *A. unedo*, 13- *P. spinosa*, 7- *R. canina* and 8- *R. micrantha*), were found in all the studied fruits. Both were positively identified according to their retention, mass and UV-vis characteristics by comparison with commercial standards. Quercetin pentoside and quercetin rhamnoside ($[M-H]^-$ at *m/z* 433 and 447, respectively) were also found in all the samples (peaks: 8 and 9- *A. unedo*; 17 and 19- *P. spinosa*; 9 and 11- *R. canina*; 10 and 12- *R. micrantha*). A quercetin hexoside ($[M-H]^-$ at *m/z* 463) was also found in *P. spinosa* (peak 14), *R. canina* (peak 8) and *R. micrantha* (peak 9) fruits. Other detected quercetin glycosides were peaks 9 and 12 in *P. spinosa*, both assigned to quercetin pentosyl-hexosides ($[M-H]^-$ at *m/z* 595); peaks 10 and 16 in *P. spinosa* ($[M-H]^-$ at *m/z* 609), tentatively identified as quercetin rhamnosyl-hexoside and quercetin hexosyl-rhamnoside; peaks 20 and 21 in *P. spinosa*, which were assigned to a quercetin acetylhexoside (pseudomolecular ion $[M-H]^-$ at *m/z* 505) and quercetin acetylrutinoside (pseudomolecular ion $[M-H]^-$ at *m/z* 651); and peaks 5 and 6 in *R. canina* and *R. micrantha* ($[M-H]^-$ at *m/z* 477), respectively, identified as quercetin glucuronides. Peaks 1 and 2 in *A. unedo* fruits were assigned to two quercetin galloylhexoside derivatives. Their identities were assigned based on their pseudomolecular ions and MS^2 spectra, releasing fragments corresponding to quercetin (*m/z* at 301) and to distinct losses of hexosyl (-162 mu), pentosyl (-132 mu), rhamnosyl (-146 mu), glucuronide (-176 mu), rutinoside (-308 mu), acetyl (-42 mu) and galloyl (-152 mu) moieties. In none of them the identity of the sugar and positions of location of the substituent could be established.

Other detected flavonols corresponded to kaempferol, isorhamnetin and myricetin derivatives. Kaempferol 3-*O*-rutinoside (peak 15 in *P. spinosa*) and isorhamnetin 3-*O*-rutinoside (peaks: 8- *P. spinosa*, 10- *R. canina* and 11- *R. micrantha*) were identified in accordance with their retention, mass spectra and UV-vis characteristics by comparison with commercial standards. Peak 5 in *A. unedo* ($[M-H]^-$ at *m/z* 447) was identified as a

kaempferol hexoside, and peaks 12 and 13 in *R. canina* and *R. micrantha*, respectively, were identified as kaempferol rhamnosyl-hexosides ([M-H]$^-$ at *m/z* 593). Two myricetin rhamnosides ([M-H]$^-$ at *m/z* 463) were also found in *A. unedo* (peaks 3 and 7) releasing an MS^2 fragment at *m/z* 317 ([M-H-146]$^-$, myricetin, loss of a rhamnosyl moiety).

Peak 7 in *P. spinosa* fruits presented a λ_{max} around 346 nm, which could be associated to a flavonol, and a pseudo molecular ion [M-H]$^-$ at *m/z* 739, presenting a fragment at *m/z* 317 that could correspond to myricetin, so that it was tentatively identified as a myricetin derivative. Peak 5 in this fruit was assigned to a flavone, apigenin pentoside ([M-H]$^-$ at *m/z* 401), releasing an MS^2 fragment at *m/z* 269 ([M-H-132]$^-$, apigenin, loss of a pentosyl moiety).

Other flavonoids detected in *R. canina* and *R. micrantha* were identified as taxifolin (flavanonol) and eriodictyol (flavanone) derivatives, λ_{max} around 290 nm. Peaks 1 and 6 in *R. canina* and 2 and 7 in *R. micrantha* presented a pseudo molecular ion [M-H]$^-$ at *m/z* 435, releasing a fragment at *m/z* 303 [taxifolin-H]$^-$ (-132 mu, loss of a pentosyl moiety) and were tentatively identified as taxifolin pentosides. Peaks 2 and 3 *R. canina* and 3 and 4 in *R. micrantha* presented a pseudo molecular ion [M-H]$^-$ at *m/z* 449, releasing a fragment at *m/z* 287 [eriodictyol-H]$^-$ (-162 mu, loss of a hexosyl moiety) and were tentatively identified as eriodictyol hexosides.

Prunus spinosa fruits presented the highest concentration in phenolic acids (29.78mg/100 g), especially due to 3-*O*-caffeoylquinic acid, the most abundant phenolic acid, and flavone/ols (57.48mg/100 g), in which quercetin 3-*O*-rutinoside (15.63mg/100 g) was the main compound. *Arbutus unedo* fruits presented quercetin 3-*O*-glucoside as the majority flavonol (2.34mg/100 g).

Taxifolin pentoside was the majority flavanonol derivative in *R. canina* and *R. micrantha* (1.18 and 2.68mg/100 g, respectively). *Rosa micrantha* (11.16mg/100 g) presented higher amount of total flavonoids (flavonols, flavanone and flavanonol) compared to *R. canina* (5.50mg/100 g).

3.3.1.2. *Flavan-3-ols and galloyl derivatives*

Flavan-3-ols (i.e., catechins and proanthocyanidins) were other relevant flavonoids found in *A. unedo*, *R. canina* and *R. micrantha* fruits. Peak 8 in *A. unedo*, peak 11 in *R. canina* and peak 10 in *R. micrantha* were identified as (+)-catechin by comparison of its UV spectra and retention time with a commercial standard. Peak 6 in *R. canina* and Peak 7 in

R. micrantha presented a pseudomolecular ion $[M-H]^-$ at *m/z* 451, releasing an MS^2 fragment at *m/z* 289 ($[M-H-162]^-$, loss of an hexosyl moiety), corresponding to a catechin monomer. This compound was tentatively identified as (+)-catechin-*O*-hexoside, identity that was coherent with its earlier elution (higher polarity) compared with its parent aglycone. Peak 5 in *A. unedo*, peak 7 in *R. canina* and peak 8 in *R. micranta* were identified as procyanidin dimer B1 by comparison of its UV spectra and retention time with a standard available in the laboratory. Similarly, peak 8 in *R. canina* was identified as procyanidin dimer B3. Other signals at *m/z* 577, 865 and 1153, in *A. unedo* (peaks 6, 7, 10 and 11), *R. canina* (peaks 9 and 12) and *R. micrantha* (peaks 12, 17 and 18) can be respectively associated to B-type procyanidin dimers, trimers and tetramers (*i.e.*, (epi)catechin units with C4-C8 or C4-C6 interflavonoid linkages). Peak 3 in *A. unedo* (pseudomolecular ion $[M-H]^-$ at *m/z* 593) was coherent with a proanthocyanidin dimer consisting of one (epi)gallocatechin unit and one (epi)catechin unit. Peaks 13 and 15 in *R. micrantha* ($[M-H]^-$ at *m/z* 851) were coherent with proanthocyanidin trimers formed by two (epi)catechin units and one (epi)afzelechin unit. Pseudomolecular ions $[M-H]^-$ at *m/z* 739, 1027, 1189 and 1351 in *R. canina* (peaks 1, 2, 5 and 10) and in *R. micrantha* (peaks 1, 2, 5, 6, 9 and 16) could correspond to procyanidin dimers or trimers having one to three sugar units attached to them. Also, peaks corresponding to double-charged deprotonated ions $[M-2H]^{2-}$ at *m/z* 819 were detected in *R. canina* (peaks 3 and 4) and *R. micrantha* (peaks 4, 5 and 11), which could be interpreted as procyanidin tetramers bearing three hexosyl residues in accordance with previous observations by Salminen et al. (2005). Other compounds detected in the chromatograms obtained for flavan-3-ol analysis were identified as different galloyl derivatives according to their pseudomolecular ions and MS^2 fragmentation behaviour (**Table 13**). Peaks 1, 2, 4, in *A. unedo* presented pseudomolecular ions $[M-H]^-$ at *m/z* 343, 331 and 325, respectively, releasing a fragment at *m/z* 169 [gallic acid–$H]^-$ (-174, -162 and -156 mu, losses of quinic acid, hexosyl and shikimic acid moieties). These compounds were tentatively identified as galloylquinic acid, galloylhexoside and galloylshikimic acid. Other signals at *m/z* 495 and 477 in *A. unedo* (peaks 9, 12 and 14) can be respectively associated to digalloylquinic and digalloylquinic shikimic acids, respectively. These galloyl derivatives were already described in *A. unedo* by by Pawlowska et al. (2006), Mendes et al. (2011) and Tavares et al. (2010). Peak 13 in *A. unedo* showed a spectra UV similar to ellagic acid, and was identified as an ellagitannin. This compound was assigned as strictinin ellagitannin according to its pseudomolecular ion ([M-H]- at *m/z* 633) and fragmentation pattern as

described by Fortalezas et al. (2010), Tavares et al. (2010) and Mendes et al. (2011). Peak 1 in *R. micrantha* (**Table 12**) presented λ_{max} around 278 nm and was identified as methyl gallate hexoside ($[M-H]^-$ at *m/z* 345). A similar compound was already reported in rose hip extracts by Hvattum (2002) and further identified by Fecka (2009) as methylgallate 3-*O*-β-glucoside. (+)-Catechin was the most abundant flavan-3-ol found in *A. unedo* (13.51mg/100 g) and *R. canina* (3.59mg/100 g), and also was a prominent compound in *R. micrantha* (2.90mg/100 g), although a glycosylated proanthocyanidin dimer (peak 6, 4.93mg/100 g) was the most abundant flavan-3-ol in this fruit. *A. unedo* fruits presented the highest concentration in proanthocyanidins (36.30mg/100 g dw).

3.3.1.3. *Anthocyanins*

The anthocyanin profile obtained for *P. spinosa* fruits, consisting of eight compounds, was more complex that those found in the other fruits. Only one anthocyanin was detected in *R. canina* and *R. micrantha* fruits, whereas three anthocyanins were detected in *A. unedo* fruits. The analytical characteristics, identities and concentrations of the anthocyanins found in the different samples are presented in **Table 14**. Cyanidin 3-*O*-glucoside, peonidin 3-*O*-glucoside and delphinidin 3-*O*-glucoside were positively identified by comparison with standards. The identity of cyaniding 3-*O*-rutinoside and peonidin 3-*O*-rutinoside were also confirmed by comparison of their chromatographic and UV and mass spectral characteristics with data in our library. Peaks 5 and 6 of *P. spinosa* and peak 3 of *A. unedo* were assigned as cyaniding 3-*O*-pentoside and peonidin 3-*O*-pentoside, respectively, based on their mass spectra, which showed an MS^2 signal at *m/z* 287 (cyanidin; $[M-132]^+$, loss of a pentosyl moiety) and *m/z* 301 (peonidin; $[M-132]^+$, loss of a pentosyl moiety). Peaks 7 and 8 presented pseudomolecular ions $[M-H]^+$ at *m/z* 491 and 505 releasing MS^2 fragments at *m/z* 287 and 301 (cyanidin and peonidin, respectively; $[M-H-42-162]^+$, loss of an acetylhexoside moiety), and were tentatively identified as cyaniding 3-*O*-acetylglucoside and peonidin 3-*O*-acetylglucoside, respectively. Cyanidin 3-*O*-glucoside was found in all the samples, being the major anthocyanin in most of them, with exception of *P. spinosa* fruits in which cyaniding 3-*O*-rutinoside and peonidin 3-*O*-rutinoside were the major anthocyanins. These fruits also presented the highest anthocyanin concentrations, which was coherent with their higher pigmentation.

3.3.2. Comparison with literature results

The chemopreventive effect of polyphenols has often been associated with their antioxidant activity. For example, chlorogenic acids are responsible for beneficial roles of some medicinal plants in the prevention of certain oxidative diseases (Morishita & Ohnishi, 2001). Quercetin is also a very efficient antioxidant (Pietta, 2000) and appears to be active in many diseases such as cancer (Choi et al., 2001), cardiovascular (Yoshizumi et al., 2001), and neurodegenerative (Schroeter et al., 2001) disorders. Derivatives of both these compounds were abundant in the studied fruits. A range of health related properties are also reported for flavon-3-ols namely antiviral (Shahat et al., 2002), insulin-like (Anderson et al., 2004), antitumor (Miura et al., 2008), anti-inflammatory (Terra et al., 2007), and antioxidant (Maldonado et al., 2005) activities. B-type trimer showed higher antioxidant activity compared to higher oligomers (Shahat et al., 2002). Compared to resveratrol and ascorbic acid, an A-type procyanidin dimer and trimer had similar or better radical scavenging abilities (Maldonado et al., 2005). Thus, both A- and B-type procyanidins have been reported to exert antioxidant activity. Anthocyanins have demonstrated that, in addition to their colorful characteristics, they possess some positive therapeutic effects, mainly linked with their strong antioxidant properties (Wang et al., 1997). Cyanidin-3-*O*-glucoside (kuromanin) has been found to have the highest oxygen radical absorbance capacity (Wang et al., 1997). This compound is the most ubiquitous and it represents the main anthocyanin in the edible parts of several plants (Dugo et al., 2001), being also found in the four fruits studied herein. The highest phenolic acids (29.78mg/100 g), flavone/ols (57.48mg/100 g) and anthocyanins (100.40µg/100 g) contents were found in *Prunus spinosa* fruits, despite no flavan-3-ols compounds were detected in this sample. As far as we know, there is no information on the phenolic composition of *R. micrantha* fruits; our group has already characterized the phenolic compounds of flowers from this species (Barros et al., 2013). The total amounts of each phenolic group has been reported for *P. spinosa* fruits from Spain (Ganhão et al., 2010), but the individual profile of this species has not been previously described. Ganhão et al. (2010) also analysed total phenolic compounds in *A. unedo* and *R. canina*. Otherwise, for *A. unedo* and *R. canina*, there are a few publications on their individual profile of phenolic composition but from other countries (Hvattum, 2002; Pawlowska et al., 2006; Pallauf et al., 2008; Fecka, 2009; Fortalezas et al., 2010; Tavares et al., 2010; Mendes et al., 2011; Tumbas et al., 2012). *R. canina* fruits from Portugal presented some

similarities in the phenolic composition to samples from Norway, Poland and Serbia (Hvattum, 2002; Fecka, 2009; Tumbas et al., 2012). Tumbas et al. (2012) identified in the Serbian sample different phenolic acids (gallic, protocatechuic, caffeic, syringic, coumaric, vanillic, ferulic and ellagic acids) and flavonoid aglycones, such as quercetin, kaempferol and myricetin. In the Portuguese sample these types of flavonols were found but all of them were glycosylated; this could be due to the fact that the extract studied by Tumbas et al. (2012) was an infusion of rosehips, and hydrolyses of the sugar moieties could have occurred. The sample from Serbia presented a lower concentration in phenolic compounds and quercetin (296.5μg/kg of rosehip tea) was the major compound. As for the samples studied herein, (+)-catechin (3.59mg/100 g dw) was the major phenolic compound found. Samples of *R. canina* from Poland (Fecka, 2009) presented a higher concentration of phenolic compounds than the one studied here, being methylgallate-3-*O*-β-glucoside and catechin the main phenolic compounds. Furthermore, Fecka (2009) identified the presence of ellagitannins such as tellimagrandin I and II and rugosin A, B, D and E, which were not detected in our study. No quantitative information was provided in the study described by Hvattum (2002) regarding phenolic compounds of *R. canina* sample from Norway. Nevertheless, its phenolic profile was similar to the Portuguese sample here analysed, despite some differences in the identification of flavan-3-ols. For *A. unedo* fruits there are three different studies on the individual phenolic profile of samples from Italy, Spain and Portugal (Pawlowska et al., 2006; Pallauf et al., 2008; Fortalezas et al., 2010; Tavares et al., 2010; Mendes et al., 2011) and they were all very similar to the one obtained in this work, with exception of Italian *A. unedo* fruits (Pawlowska et al., 2006), for which only gallic acid derivatives and anthocyanins were reported, but not flavonols. The comparison of phenolic compounds quantification was not possible, due to the fact that these authors only quantified the anthocyanins, expressing the results in fresh weight. However, the amounts obtained for Spanish *A. unedo* fruits (Pallauf et al., 2008) were very similar to ours. Mendes et al., 2011, Tavares et al., 2010 and Fortalezas et al., 2010 studied a different sample from Portugal, but did not present any type of quantification.

Previous *in vitro* chemical and biochemical assays demonstrated that the studied fruits have a high antioxidant activity and that this could be correlated to their phenolic composition (Barros et al., 2010; Guimarães et al., 2010; Barros et al., 2011). Overall, *Prunus spinosa* fruits presented the highest concentration in phenolic acids and

flavone/ols, being 3-*O*-caffeoylquinic acid and quercetin 3-*O*-rutinoside the major compounds. (+)-Catechin was the most abundant compound in *Arbutus unedo* and *Rosa canina* fruits. *A. unedo* fruits presented the highest concentration in flavan-3-ols. Cyanidin 3-*O*-glucoside was found in all the studied fruits, being the major anthocyanin in most of them, with the exception of *P. spinosa,* were cyaniding 3-*O*-rutinoside and peonidin 3-*O*-rutinoside predominated. All in all, *P. spinosa* presented the highest levels of phenolic acids and flavonoids, including anthocyanins, flavonols and flavones, although no flavan-3-ols could be identified in its fruits. The present study represents a contribution to the chemical characterization of phenolic extracts from wild fruits with reported antioxidant activity and traditionally used for several medicinal applications. The studied fruits may have great potential for food industries as a source of colors and flavors, as well as bioactive molecules such as phenolic compounds for dietary supplements or functional foods.

3.3.3. Antioxidant and antitumor activity

The results of antioxidant activity, determined by free radicals scavenging activity, reducing power and inhibition of lipid peroxidation in brain cell homogenates, are shown in **Table 15**.

Table 15. Antioxidant activity[a] of different phenolic enriched extracts from four wild fruits (mean ± SD).

	DPPH scavenging Activity	Reducing Power	β-carotene bleaching inhibition	TBARS inhibition
PE				
Arbutus unedo	60.89±1.74^{d}	36.69 ± 1.82^{c}	432.08±19.37^{c}	7.21 ± 0.35^{c}
Prunus spinosa	64.98 ± 6.19^{c}	42.08 ± 0.66^{b}	641.11 ± 80.69^{b}	7.39 ± 0.20^{c}
Rosa canina	75.78 ± 4.10^{a}	47.38 ± 0.93^{a}	852.20 ± 147.67^{a}	10.02±0.29^{a}
Rosa micrantha	69.58 ± 3.37^{b}	47.80 ± 1.19^{a}	755.39 ± 82.25^{b}	8.89 ± 0.26^{b}
	DPPH scavenging Activity	Reducing Power	β-carotene bleaching inhibition	TBARS inhibition
AE				
Arbutus unedo	93.75 ± 2.26^{b}	75.41±0.53^{b}	950.96 ± 38.71^{a}	23.13±3.21^{b}
Prunus spinosa	99.37 ± 2.36^{a}	83.30 ± 0.46^{a}	n.a.	25.29 ± 0.85^{a}
Rosa canina	81.21±2.26^{d}	72.75±2.38^{c}	893.57±29.19^{b}	12.39 ± 0.18^{c}
Rosa micrantha	86.33±1.69^{c}	75.25±0.12^{b}	904.08±55.50^{b}	22.52±0.36^{b}
Trolox	43.03 ± 1.71	29.62 ± 3.15	2.63 ± 0.14	3.73 ± 1.90

[a]EC_{50} values (μg/mL) corresponding to the sample concentration achieving 50% of antioxidant activity or 0.5 of absorbance in reducing power assay. n.a. It was not possible to obtain EC_{50} value for this extract. PE- Non-anthocyanin phenolic compounds enriched extract; AE- Anthocyanins enriched extract. In each column, and for each extract, different letters mean significant differences ($p<0.05$).

The studied extracts were chemically characterized in a previous work of our research group (Guimaraes et al., 2013). Herein, two different enriched phenolic extracts were prepared, in order to evaluate and compare their bioactivity: a non-anthocyanin phenolic compounds enriched extract (PE; with phenolic acids, flavones/ols, flavan-3-ols and galloyl derivatives) and a separate anthocyanins enriched extract (AE).

Regarding PE of the studied wild fruits, *A. unedo* presented the highest antioxidant activity in all the *in vitro* assays, which could be related to the presence of galloyl derivatives (exclusively in *A. unedo* PE) and/or to the presence of higher levels of flavan-3-ols. The second one with highest antioxidant effects was *P. spinosa*, in which the main contributors seemed to be phenolic acids (exclusive in *P. spinosa* PE) and flavones/ols, present in this PE in higher amounts. The studied Rosa species revealed the lowest antioxidant activity, presenting similar phenolic compounds profile (flavan-3-ols and flavones/ols); the higher levels of these compounds found in *R. micrantha* comparatively to *R. canina*, might explain the higher antioxidant activity observed in the first case (**Table 15**, **Figure 13**).

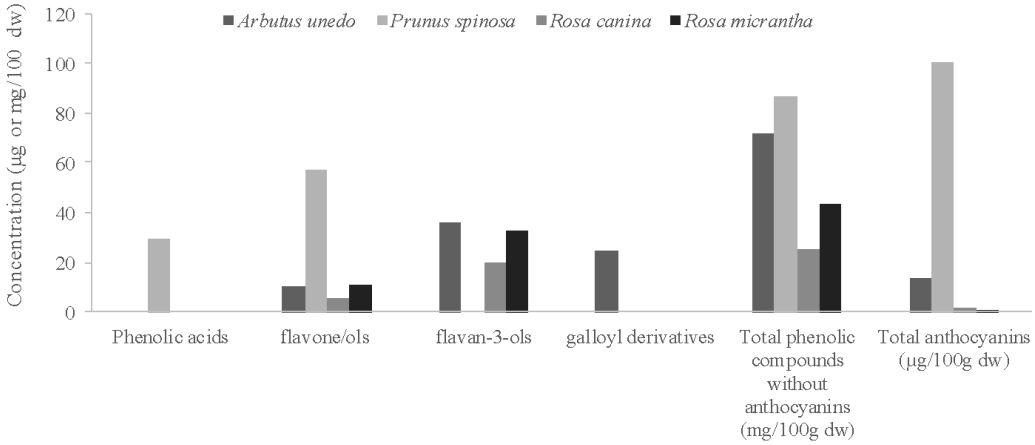


Figure 13. Concentrations of phenolic compounds present in the wild fruits, determined by HPLC-DAD-MS/ESI according to reference (Guimarães et al., 2013).

Concerning AE, a pro-oxidant effect of anthocyanins seemed to occur, since the samples with the highest amounts revealed the lowest antioxidant activity (**Table 15**, **Figure 13**). For that reason, *P. spinosa* gave the lowest antioxidant activity (in β-carotene-bleaching inhibition assay it was not possible to determine EC_{50} value due, in our opinion, to pro-

oxidant effects of anthocyanins), while *R. canina* showed the highest antioxidant effects.

PE gave higher antioxidant properties than the corresponding AE, and according to their chemical characterization, those prperties seem to be related to galloyl derivatives, flavan-3-ols, phenolic acids and flavones/ols. In general, PE and AE presented higher antioxidant activity than the methanolic extracts (crude extracts) of the same fruits previously studied by us (Barros et al., 2010; Guimaraes et al., 2010). It seems that purified/ enriched extracts (such as the cases herein presented) are more suitable than crude extracts, in wich antagonistic effects between the compounds presente could be observed, conducting to a decrease in the antioxidant activity. The only exception was for de β-carotene bleaching inhibition assay (higher capacity in crude extracts); in this case, other molecules rather than the ones previously mentioned are probably involoved and the might bring synergistic effects.

The antitumor potential was tested in human tumor cell lines (breast, lung, colon, cervical and hepatocellular carcinomas), and the hepatotoxicity was evaluated using a porcine liver primary cell culture. All the extracts inhibited the growth of tumor cell lines, except *R. canina* PE and AE, *P. spinosa* AE and *R. micrantha* AE for MCF-7 (breast carcinoma). *A.unedo*, followed by *P. spinosa*, PE gave the best antitumor inhibition (**Table 16**), which could be correlated as mentioned above for antioxidante activity (similar behaviour), to the phenolic groups present in each of the wild fruits (**Figure 13**), i.e, exclusive presence of galloyl derivatives and the highest levels of flavan-3-ols for *A. unedo* PE, and the exclusive presence of phenolic acids and the highest levels of flavones/ols for *P. spinosa* PE. Regarding AE, samples with the highest amounts of anthocyanins (*P. spinosa and A. unedo*) revealed the highest antitumor effects, except in the case of MCF-7 that was not inhibited by *P. spinosa* AE. None of the samples showed toxicity for non-tumor liver primary culture.

Table 16. Antitumor activity and hepatotoxicity[a] of different phenolic enriched extracts from four wild fruits (mean ± SD).

PE	MCF-7 (breast carcinoma)	NCI-H460 (non-small lung cancer)	HCT-15 (colon carcinoma)	HeLa (cervical carcinoma)	HepG2 (hepatocellular carcinoma)	PLP2 (non-tumor liver primary culture)
Arbutus unedo	153.08±10.34^{d}	37.68±5.02^{d}	93.36±5.98^{c}	143.36±9.07^{d}	128.51±7.47^{d}	>400
Prunus spinosa	270.65±9.25^{c}	154.25±6.35^{c}	220.44±2.89^{b}	193.62±11.05^{c}	169.56±6.39^{c}	>400
Rosa canina	>400^{a}	254.69±3.91^{a}	243.67±4.65^{a}	253.03±11.03^{a}	281.79±5.78^{a}	>400
Rosa micrantha	374.11±8.69^{b}	226.04±7.56^{b}	223.25±4.23^{b}	226.34±13.81^{b}	255.31±9.01^{b}	>400
AE	MCF-7 (breast carcinoma)	NCI-H460 (non-small lung cancer)	HCT-15 (colon carcinoma)	HeLa (cervical carcinoma)	HepG2 (hepatocellular carcinoma)	PLP2 (non-tumor liver primary culture)
Arbutus unedo	238.11±6.74	227.43±4.09^{c}	121.95±7.15^{b}	149.88±8.46^{d}	168.40±7.29^{c}	>400
Prunus spinosa	>400^{a}	282.92±4.28^{b}	234.59±6.29^{a}	224.58±14.09^{c}	231.31±9.24^{b}	>400
Rosa canina	>400^{a}	305.97±4.23^{a}	243.51±8.24^{a}	311.16±10.18^{a}	266.53±10.95^{a}	>400
Rosa micrantha	>400^{a}	307.68±6.05^{a}	264.04±3.08^{a}	252.07±11.01^{b}	270.5±9.24^{a}	>400
Ellipticine	0.91±0.04	1.42±0.00	1.91±0.06	1.14±0.21	3.22±0.67	2.06±0.03

[a]GI_{50} values (μg/mL) corresponding to the sample concentration achieving 50% of growth inhibition in human tumor cell lines or in liver primary culture PLP2. PE- Non-anthocyanin phenolic compounds enriched extract; AE- Anthocyanins enrichedextract. In each column, and for each extract, different letters mean significant differences ($p<0.05$).

As far as we know, thisb is the first study regarding antitumor effects of *A. unedo*, *R. micrantha* and *P. spinosa* wild fruits. In the case of *R. canina*, the antitumor effects of na aqueous extract from its hips were studied in mouse melanoma cells (Fujii et al., 2011), and similarly to the herein studied PE, the higher contributors are proanthocyanidins (flavan-3-ols). Otherwise, the flavonoids fraction from *R. canina* tea showed higher antiproliferative activity in HeLa cell line (IC_{50} = 80.63 µg/mL; Tumbas et al., 2012) than the one observed in the present study for PE (GI_{50} = 253.03 µg/mL), contrarily to the observed result (no activity up to 400 ug/mL), those authors reported effects against MCF-7 cell line (IC_{50} = 248.03 µg/mL).

Overall, the bioactivity of the studied wild fruits proved to be more related to phenolic compounds profile than to the amounts present in each extract being PE more bioactive than AE. It should be highlighted the high capacity of *A. unedo* PE to inhibit lipid peroxidation in animal brain homogenates (IC_{50} = 7.21 µg/mL), as also its antitumor potential against NCI-H460 human cell line (non-small lung cancer; GI_{50} = 37.68 µg/mL). Regarding chemical characterization of the mentioned sample, the presence of galloyl derivatives exclusively in *A. unedo* wild fruits could be related to its higher bioactivity. Further studies are needed in order to confirm the specific role of these compounds in antioxidante and antitumor effects. Due to the observed bioactive properties, the mentioned species could be considered in the design of new formulations of dietary supplements or functional foods.

Chapter 4.
Chemical Synthesis

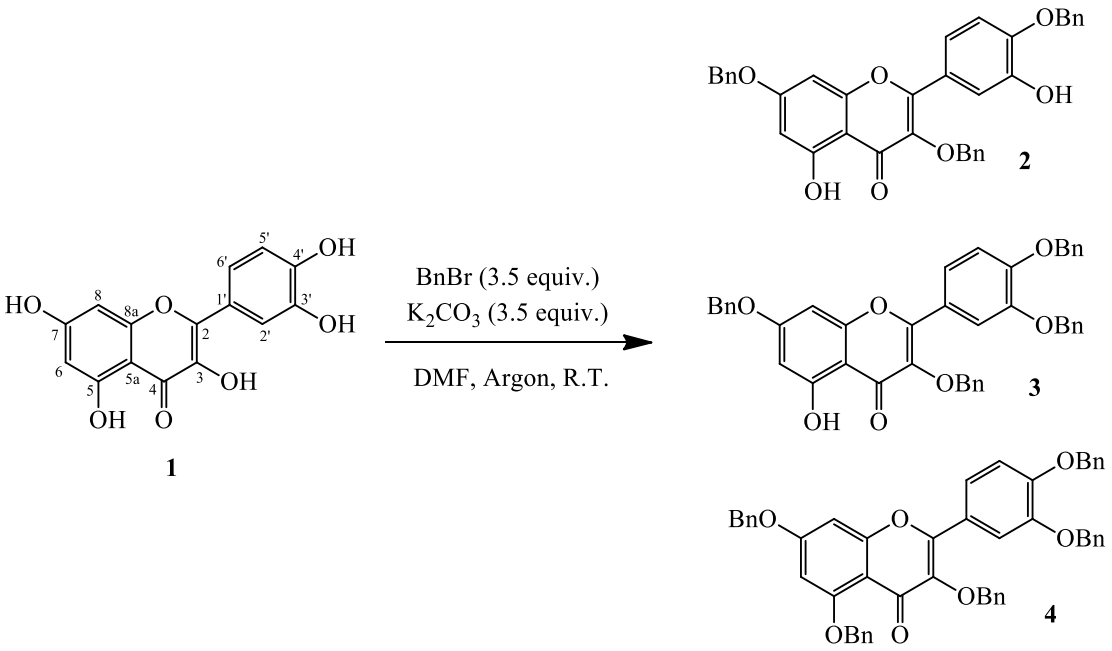
Chapter 4. Chemical Synthesis

Flavonoids are present in a great variety of food and especially in fruit and vegetables. Quercetin is the flavonoid most swallowed every day (between 10 and 100/mg per day according food habits) (Kajjout & Rolando, 2011). Moreover, it was identified in the studied plant species. Quercetin is a very efficient antioxidant (Pietta, 2000) and appears to be active in many diseases (Choi et al., 2001; Yoshizumi et al., 2001; Schoroeter et al., 2001). In blood, quercetin is mainly found in conjugated forms. The main metabolic pathways in humans and mammals for this flavonol are methylation, sulphation, and glucuronidation (Manach et al., 1998; Day et al., 2001; Justino et al., 2004; Van der Woude et al., 2004; Mullen et al., 2008). The relative amount of metabolites varies either in their chemical nature or in their position on the quercetin ring, according to the species and the metabolism phase (Kajjout & Rolando, 2011). Some studies using plasma samples containing sulphate or glucuronide conjugates or both and methylated forms showed that the biological activities of these metabolites were rather different from the quercetin (Koga & Meydani, 2001).

In humans quercetin 3-*O*-β-D-glucuronide and quercetin 3'-*O*-sulphate are the most abundant quercetin metabolites and have even more potent biological activities than quercetin **1** (Chao et al., 2009). Plasma analyses of pig fed with quercetin rich diets showed that there is no quercetin left but only methylated metabolites, such us 3'-*O*-methylquercetin (isorhamnetin) and 4'-*O*-methylquercetin (tamarixetin) along with the 3'-dehydroxylated quercetin (kaempferol), most of being conjugated as glucuronide or sulphate (Ader et al., 2000). However, quercetin metabolism in humans is still controversial as the different relative abundance of metabolites depends of the diet habits and the quercetin content (Graefe et al., 1999; Graefe et al., 2001; Manach et al., 1998; Moon et al., 2001). In order to evaluate the bioactivity of some of flavonoids derivatives/human metabolites we tried the synthetic approach.

4.1. Benzylation reactions

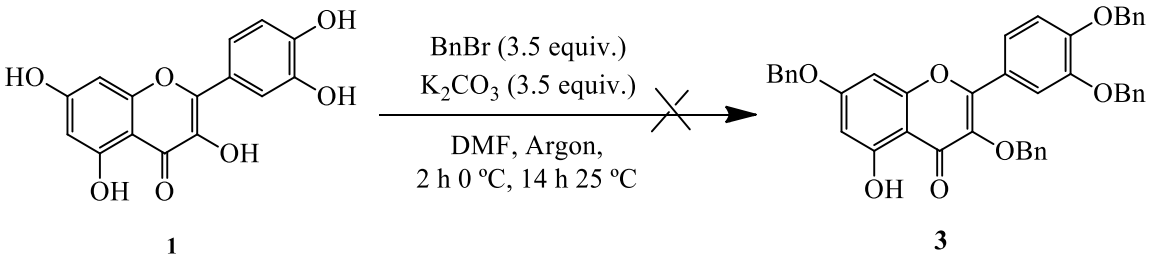
The literature mentions some possible methods of synthesis for flavonoid derivatives, especially for the most common, quercetin, using the protection of hydroxy group approach. Our work started with the benzylation of the hydroxy groups. The benzyl group was chosen as protecting group because it is easily introduced by reaction with a benzyl halide and also easily removed by hydrogenolysis (Kajjout & Rollando, 2011). Following the conditions of the Kajjout & Roland 2011, a solution of quercetin **1**, in DMF, was reacted with benzyl bromide and potassium carbonate under argon atmosphere, at room temperature (***Scheme 1***; **Experiment 1**, **Section 4.4.1.1**).



Scheme 1. Benzylation reaction of quercetin **1**.

After the work-up procedure, the crude residue was analyzed by ^{1}H-NMR spectroscopy (results not shown). The spectrum showed that the reaction should have formed a mixture of products with different protection degrees namely *O*-tri- (**2**), *O*-tetra- (**3**) and pentabenzylated quercetin (**4**) (***Scheme 1***), in good agreement with the results reported in the literature (Kajjout & Rolando, 2011). Unfortunately, it was not possible to separate the products by column chromatography, probably due to their very similar solubilities.
In view of these results it was decided to study this reaction with temperature control. Thus, all the reagents were mixed at 0 ºC, under stirring and the temperature was kept for

2 h, followed by 14 h at room temperature (to obtain a good temperature adjustment, the reaction was carried out in a Cooled Incubator MIR-154), following the conditions of Bouktaib et al., 2002 to obtain the compound **3** (***Scheme 2***, **Experiment 2**, **Section 4.4.1.2**).



Scheme 2. Attempt to obtain 3,7,3'4'-*O*-tretrabenzylated quercetina **3**.

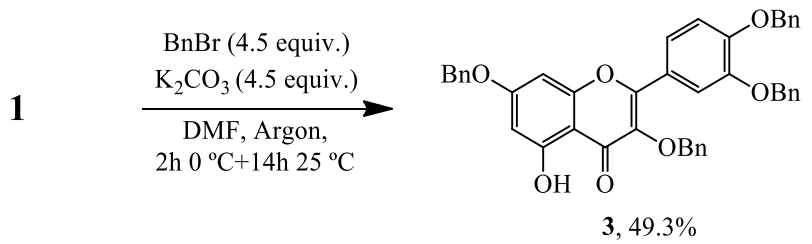
Once again, the ^{1}H-NMR spectrum of the crude residue showed that the formation of products with different degree of protection had occurred (results not shown). The purification by column chromatography, using CH_2Cl_2 as eluent, was not well succeeded, and the expected product **3** (Bouktaib et al., 2002) was not isolated.

Following these results, it was decided to investigate the introduction of the same protecting group using different amounts of the alkylating agent (BnBr) and of the base (K_2CO_3) (***Table 17***, **Experiments 3-14**, **section 4.4.1.3 and 4.4.1.4**).

Table 17. Benzylation of quercetin using different amounts of alkylating agent (BnBr).

Exp.	Solvent (dried)	BnBr (equiv.)	K_2CO_3 (equiv.)
3	DMF	3.5	1.5
4			1.75
5			2.0
6			3.0
7			3.25
8			3.5
9			4.0
10		4.0	4.0
11		4.5	4.0
12			4.1
13			4.5
14	DMSO	4.5	4.5

All reactions afforded a variety of products, but the mixtures from reactions 13 and 14 (***Table 17***), showed mainly one or two products by ^{1}H-NMR. Experiment 13 gave mainly the 3,7,3',4'-*O*-tetrabenzylated quercetin **3** (***Scheme 3***), while experiment 14, using DMSO, gave the 3,7,4'-*O*-tribenzylated quercetin **2** (***Scheme 4***).



Scheme 3. Synthesis of 3,7,3',4'-*O*-tetrabenzylated quercetina **3** using Bouktaib et al., 2002 conditions, but 4.5 equiv of BnBr and of K_2CO_3.

The ^{1}H-MNR spectrum of the reaction (***Scheme 3***, **Experiment 13**, **Section 4.4.1.3**) residue shows the presence of a major *O*-tetrabenzylated derivative with several other

benzylated derivatives with different protection degrees. This residue was washed with methanol, allowing the solubilization of the polar components and precipitation of the less polar compound. The solid isolated by filtration was identified by ^{1}H-NMR as the *O*-tetrabenzylated quercetin derivative **3** in a quite pure form (**Figure 14**), in 49.3% yield.

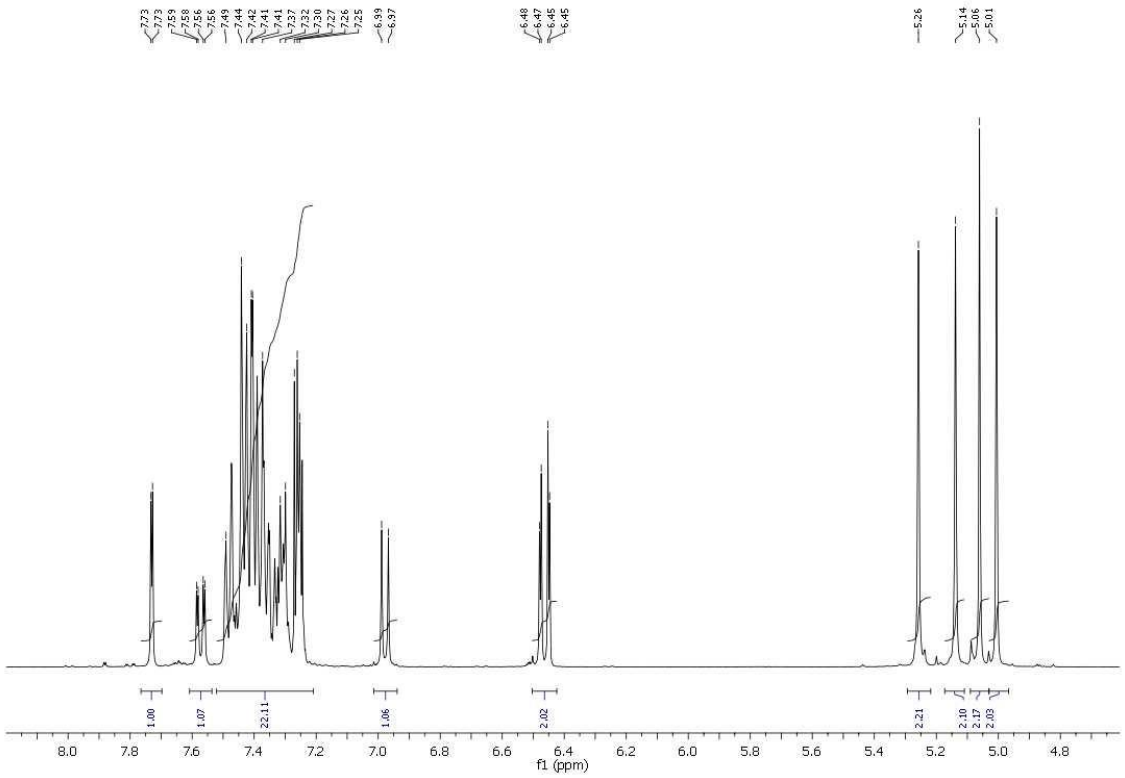
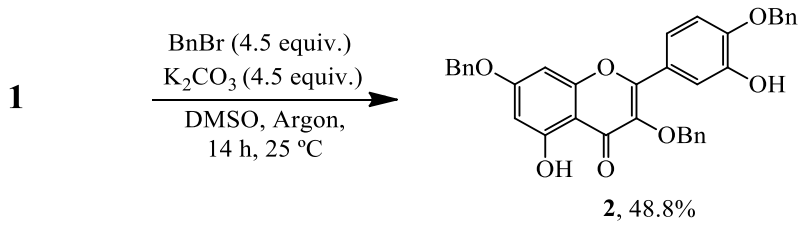


Figure 14. ^{1}H-NMR (400 MHz, $CDCl_3$) spectrum of **3** (Experiment 13, Section 4.4.1.3).

Considering the protection reactivity order 4'>7>3>3'>5 referred by Shi et al., 2012, the structure **3** is proposed for the *O*-tetrabenzylated derivative of quercetin obtained (***Scheme 3***), which ^{1}H-NMR spectrum data are similar to the ones presented in the literarture by Bouktaib et al., 2002 and Kajjout & Rolando, 2011 (**Section 4.4.1.3**).
The experiment 14 is presented in ***Scheme 4*** (**Table 17**).



Scheme 4. Synthesis of 3,7,4'-*O*-tribenzylated quercetin **2** in DMSO (Experiment 14).

The ^{1}H-NMR spectrum of the impure residue obtained from this experiment 14 showed that the mixture was less complex than those obtained before. This residue was mainly formed by a *O*-tribenzylated and a *O*-tetrabenzylated derivative in a 76:24 ratio, respectively, by ^{1}H-NMR, along with traces of other non identified benzylated derivatives. The residue was purified by recrystallization from dichloromethane:hexane and led to a yellow solid (**Experiment 14, Section 4.4.1.4)** which was identified by ^{1}H-NMR (**Figure 15**). Based on the different reactivity of the hydroxy groups of quercetin claimed by Shi et al., in 2012, 4'>7>3>3'>5, the structure **2** is proposed for the *O*-tribenzylated quercetin obtained, which ^{1}H-NMR spectrum data are similar to the ones presented in the literature by Bouktaib et al., 2002 and Shi et al., 2012 (**Experiment 14**, **Section 4.4.1.4**). In the ESI mass spectrum at low resolution a peak *m/z* 595.20 (M^{+}+Na, 17%) was observed.

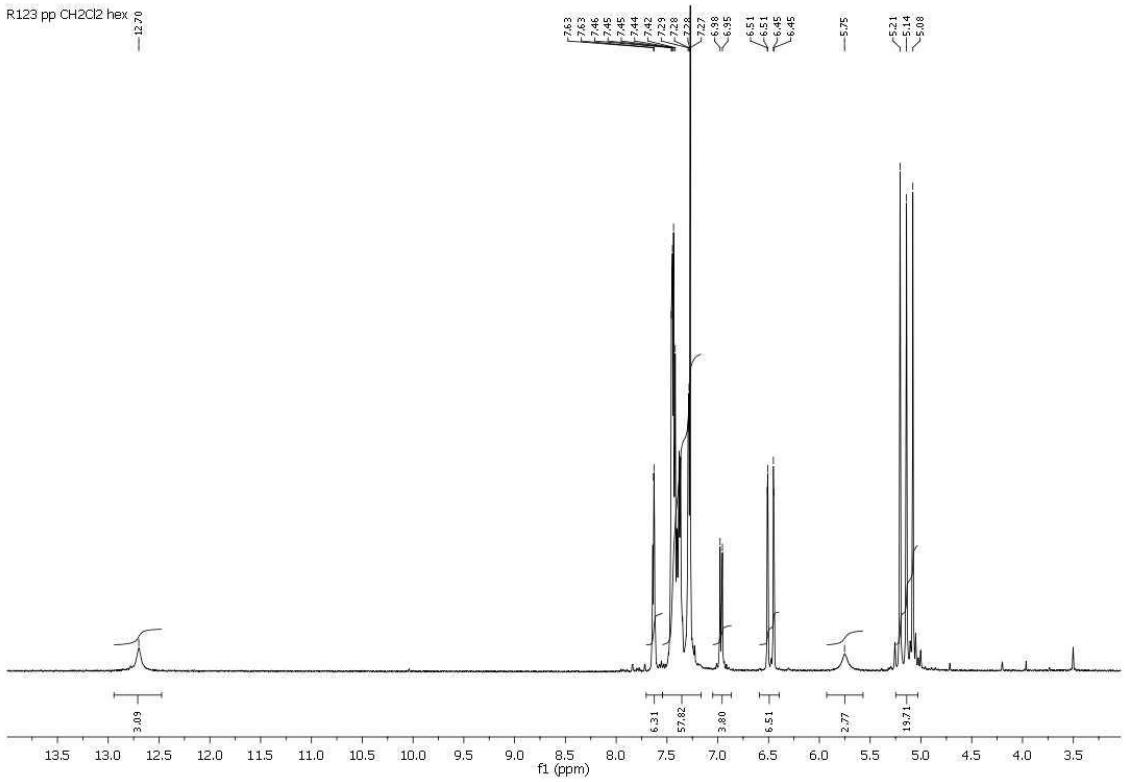
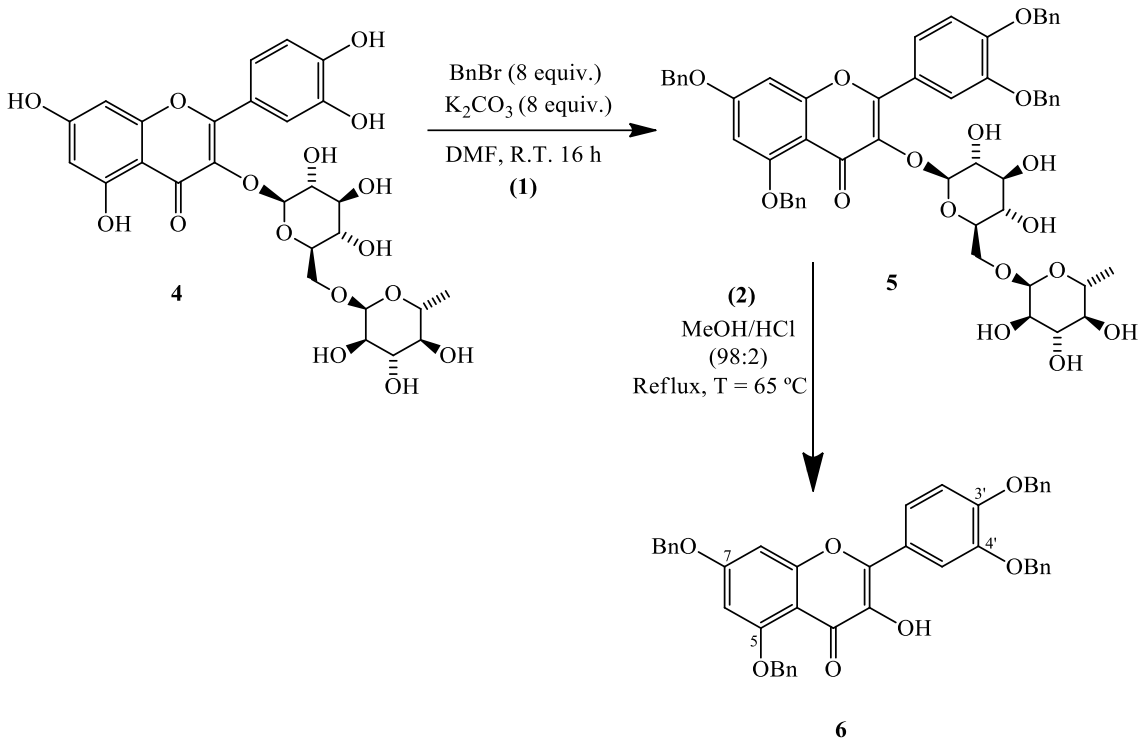


Figure 15. ^{1}H-NMR (400 MHz, $CDCl_3$) spectrum of recrystallized compound **2** (Experiment 14, Section 4.4.1.4).

In order to obtain the 5,7,3',4'-*O*-tetrabenzylated quercetin **6**, it was decided to prepare it from rutin **4** using the method described by Kajjout et al. 2011. This reaction was carried out in two steps outlined in ***Scheme 5***: (1) the four free hydroxy groups of rutin **4** were benzylated affording compound **5**;

(2) the hydrolysis of compound was performed giving the desired compound **6** (***Scheme 5***, **Experiment 15**, **Section 4.4.1.5**).



Scheme 5. Synthesis of the 5,7,3',4'-*O*-tetrabenzylated quercetina **6** from rutin **4.**

The crude residue obtained from step 1 was identified by ^{1}H-NMR as the *O*-tetrabenzylated derivative of rutin **5** according to Kajjout et al., 2011. It was used with no further purification in step 2, where a solid was formed and identified by ^{1}H-NMR spectroscopy as compound **6** in almost pure form, **Figure 16**. The NMR data are similar to those found in the literature, Kajjout et al., 2011.

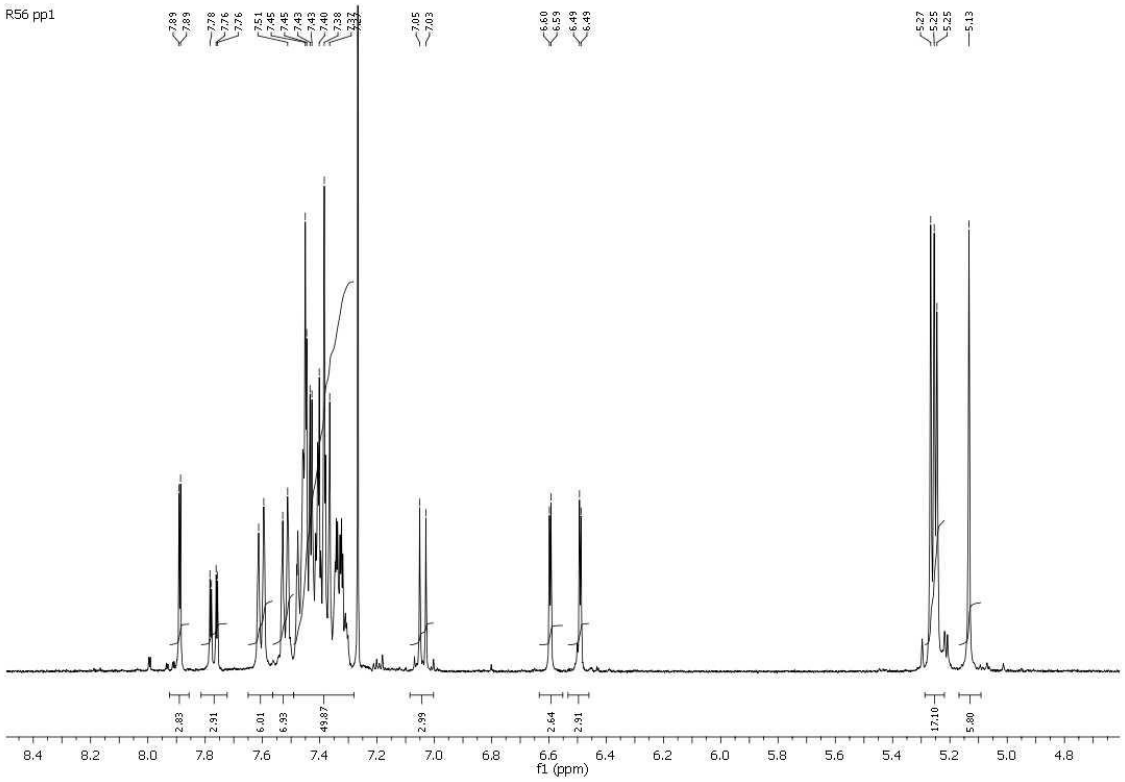
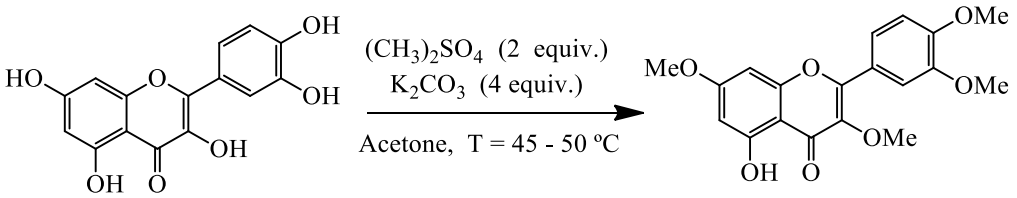


Figure 16. ^{1}H-NMR (400 MHz, $CDCl_3$) spectrum of the compound **6** (Experiment 15, Section 4.4.1.5).

In this work it was possible to obtain one *O*-tribenzylated quercetin **2**, with free hydroxy groups in positions 5 and 3', and two *O*-tetrabenzylated quercetins **3** and **6**, with free hydroxy groups at 5 or 3 positions, respectively using different synthetic strategies.

4.2. Methylation reactions

In face of the results obtained in the reactions involving the benzyl group as the protective, it was decided to investigate the protection of the hydroxy groups of quercetin with methyl group, a less bulky group. As mentioned before, it is known that one of the metabolic pathways in humans and mammals, for quercetin, involves its methylation. The *O*-tetramethylated quercetin **7** was obtained by reacting compound **1** with dimethyl sulphate and potassium carbonate, in acetone (***Scheme 6***, **Experiment 16**, **Section 4.4.2.1**) in 49% yield as a yellow solid.



Scheme 6. Synthesis of 3,7,3',4'-O-tetramethylated quercetina **7**.

The ^{1}H-NMR spectrum of compound 7 is presented in **Figure 17**.

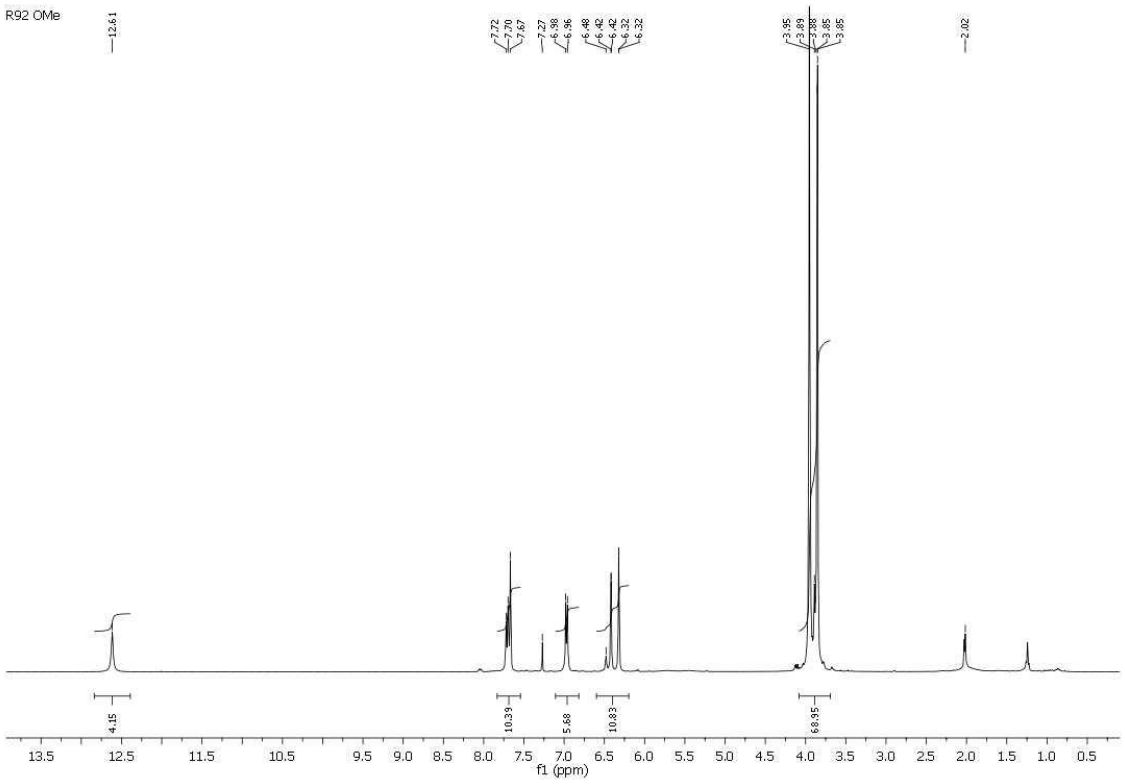
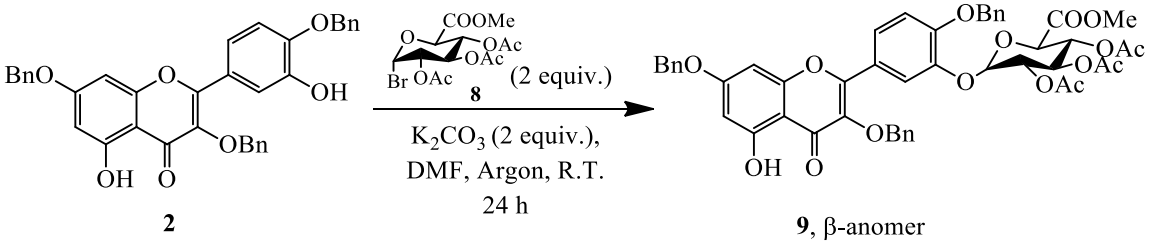


Figure 17. ^{1}H-NMR (400 MHz, $CDCl_3$) spectrum of the compound **7** (Experiment 16, Section 4.4.2.1).

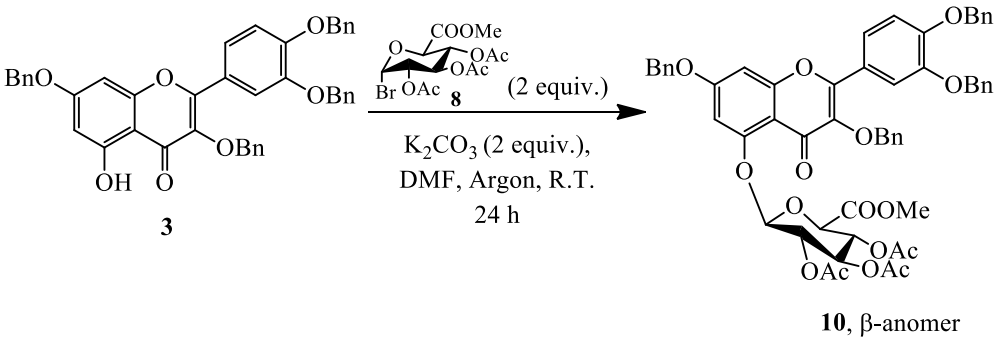
Rao et al. in 1976, have shown that the methylation occurs gradually according to the sequential order of the positions: 4'>7>3>3'>5, the less reactive hydroxy group being that of the 5 position. Based on this reactivity order, structure **7** is proposed for the *O*-tetramethylated quercetin derivative obtained (***Scheme 6***). As far as our knowledge this compound is not be described in the literature, see experiment 16.

4.3. Glucuronidation reactions

As previously mentioned, in human plasma quercetin usually appears as glucuronides. Therefore, in this work the glucuronidation of quercetin derivatives **2** (3,7,4'-*O*-tribenzylated), **3** (3,7,3',4'-*O*-tetrabenzylated) and **6** (5,7,3',4'-*O*-tetrabenzylated) was attempted by their reactions with acetobromo-α-D-glucuronic acid methyl ester **8**, according to *schemes 7, 8* and *9* (**Experiments 17**, **18 and 19, section 4.3.1.1**), respectively.



Scheme 7. Glucuronidation reaction of compound **2.**



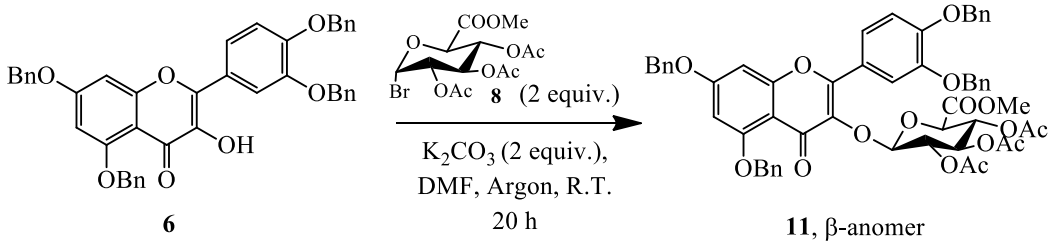
Scheme 8. Glucuronidation reaction of compound **3**

In each case the separation of these compounds (***scheme 7*** and ***8***) was attempted by column chromatography in order to obtain the desired glucuronide derivative in a pure form. Unfortunately, none of these two reactions were well succeeded. The ^{1}H-NMR

spectrum of the crude residue obtained in the reaction displayed in ***Scheme 7*** showed a complex mixture of compounds. The starting materials **2** and **8**[(1)], were present and the compound **9** expected as a β-anomer (***Scheme 7***) seemed to be present only in a small amount, based on a doublet at 5.78 ppm with J = 8.0 Hz (data not shown). The formation of this compound was corroborated by the mass spectrum obtained by ESI that showed a peak *m/z* 911.42 (M^++Na, 7.4%) (data not shown).

The reaction displayed in ***Scheme 8*** afforded also a very complex crude residue and the fractions collected after column chromatography were not possible to identify.

Starting from compound **6**, the glucuronidation reaction was attempted in order to obtain the glucuronide derivative **11** (***Scheme 9***).

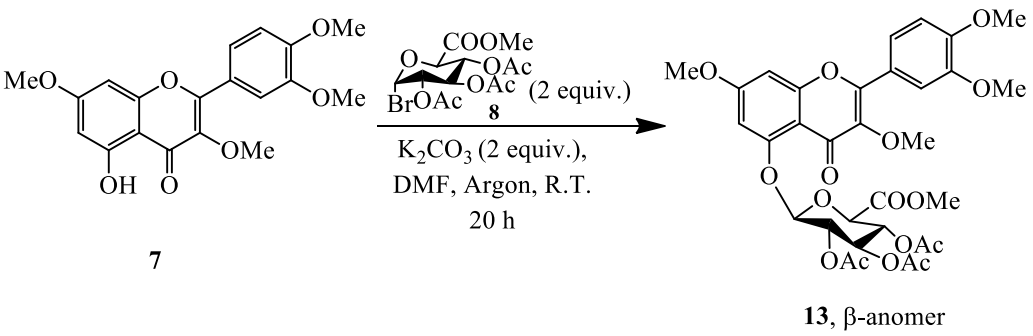


Scheme 9. Glucuronidation reaction of compound **6.**

The ^{1}H-NMR spectrum of the crude residue of experiment 19 showed a complex mixture of compounds. It was possible to identify three major signals of acetyl groups along with several other minor signals. None of them was attributed to acetyls of compound **8**. The expected β-anomer was associated with the doublet at 5.78 ppm with a coupling constant J = 8.0 Hz (data not shown). Compound **11** was effectively formed, although in a small amount, as it was confirmed by mass spectrometry analysis. The mass spectrum obtained by ESI showed a peak of *m/z* 1001.40 as the base peak (100%) which corresponds to M^++Na.

The glucuronidation of **7** (3,7,3',4'-*O*-tetramethylated quercetin) was attempted using the experimental conditions presented in ***Scheme 10*** (**Experiment 20, Section 4.4.3.2**).

(1) ^{1}H NMR (400 MHz, $CDCl_3$): 2.06, 2.07 and 2.11 (9H, 3s, 3 × OAc); 3.77 (3H, s, COOMe); 4.59 (1H, dd, 10.0 Hz, H-5); 4.86 (1H, dd, J = 10.0 and 4.0 Hz, H-2); 5.25 (1H, dd, J = 10.4 and 9.6 Hz, H-3); 5.62 (1H, apparent t, J = 9.6 Hz, H-4); 6.65 (1H, d, J = 4.0 Hz, H-1);



Scheme 10. Glucuronidation reaction of compound **7**.

The ^{1}H-NMR spectrum of the crude residue of experiment 20, revealed that a very complex mixture was formed. The presence of some signals and their multiplicity could suggest that glucuronidation had occurred although in a small extent. In fact it was possible to find a doublet at 5.77 ppm with a coupling constant J = 7.6 Hz (data not shown), that was attributed to the anomeric proton of a β-anomer. It was also found a great number of signals attributed of different acetyls and none of them correspond to the ones of starting material **8**. It was also possible to see the presence of several singlets of methyl groups. Nevertheless, this residue was submitted to column chromatography but it was not possible to separate the products.

In conclusion, these reactions of glucuronidation did not give the expected compounds in a pure form and therefore we could not study their biological activity.

4.4 Synthesis of flavonoid derivatives: Experimental Section

The numbers of the compounds are related to the ones presented in **Chapter 4**.

General procedure: The starting material flavonoids and acetobromo-α-D-glucuronic acid methyl ester, were purchased at Sigma Aldrich. Melting points (ºC) were determined in STUART SMP3 and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance III at 400 MHz. DEPT 135º experiments were used to differentiate the different type of carbons. Heteronuclear correlations ^{1}H–^{13}C, HSQC and HMBC were performed to attribute some signals. The mass spectra were obtained on a LXQ Finnigan by Electro Spray Ionization (ESI).

4.4.1 Benzylation experiments

4.4.1.1 Experiment 1 (Section 4.1, *Scheme 1*)

To a solution of quercetin **1** (0.200 g, 0.660 mmol) in DMF (3 mL), potassium carbonate (0.320 g, 2.32 mmol, 3.5 equiv.) and BnBr (0.276 mL, 2.32 mmol, 3.5 equiv.) were added under argon. The reaction mixture was vigorously stirred at room temperature for 16 h. The resulting mixture was diluted with ethyl acetate (80 mL) and washed with water (3x50 mL). The organic layer was dried ($MgSO_4$) and evaporated, affording a complex mixture (by TLC and ^{1}H-NMR spectrum). This mixture was submitted to flash column chromatography (silica gel, dichloromethane), giving products **2**, **3**, and **4**, identified in the ^{1}H-NMR spectrum (***Scheme 1***, **Section 4.1**), according to Kajjout & Rolando 2011, but were not possible to separate.

4.4.1.2 Experiment 2 (Section 4.1, *Scheme 2*)

To a solution of quercetin **1** (0.200 g, 0.660 mmol) in DMF (3 mL), potassium carbonate (0.320 g, 2.32 mmol, 3.5 equiv.) and BnBr (0.276 mL, 2.32 mmol, 3.5 equiv.) were added under argon. After vigorous stirring at 0 ºC for 2 h, the reaction mixture was allowed to warm to room temperature and the stirring was maintained for 14 h. The resulting mixture was diluted with iced water (50 mL), extracted with ethyl acetate (3 x 50 mL). The organic layer was washed with water (3 x 80 mL), dried ($MgSO_4$) and evaporated, affording a complex mixture that was submitted to flash column chromatography (silica gel, dichloromethane), but the products formed was not possible to be separated (***Scheme 2***, **Section 4.1**).

4.4.1.3 Experiments 3-13 (*Section 4.1*)

All these experiments were carried out under the experimental conditions described in the Experiment 2, changing the equivalents of benzyl bromide and/or potassium carbonate as shown in the **Table 17 (Chapter 4, Section 4.1)**.

In Experiment 13 the washing up of the residue with methanol allowed the solubilization of more polar compounds and the precipitation of the less polar. This solid was isolated by filtration (0.225 g, 49.3 % yield) and as a yellow solid identified as **3 (Section 4.1,**

***Scheme 3*)**. MP 137–139 ºC [140-142 ºC – Bouktaib et al., 2002 and Kajjout & Rolando, 2011]. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.01 (s, 2H, 3'-OCH_2Ph), 5.06 (s, 2H, 3-OCH_2Ph) 5.14 (s, 2H, 7-OCH_2Ph), 5.26 (s, 2H, 4'-OCH_2Ph), 6.45 (d, *J* = 2.0 Hz, 1H, H-6), 6.48 (d, *J* = 2.0Hz, 1H, H-8), 6.98 (d, *J* = 8.8 Hz, 1H, H-5'), 7.25-7.49 (m, 20H, 4 x Ph), 7.57 (dd, *J* = 8.4 and 2.0 Hz, 1H, H-6'), 7.73 (d, *J* = 2.0 Hz, 1H, H-2'), 12.72 (1H, broad s, OH) ppm. ^{13}C NMR (100.6 MHz**,** $CDCl_3$): δ = 70.40 (7-OCH_2Ph), 70.82 (4'-OCH_2Ph), 71.05 (3'-OCH_2Ph), 74.31 (3-OCH_2Ph), 93.01 (C8), 98.53 (C6), 106.12 (C5a), 113.63 (C5'), 115.24 (C2'), 122.58 (C6'), 123,40 (C1'), 127.15 (CH), 127.31 (CH), 127.43 (CH), 127.87 (CH), 127.99 (CH), 128.25 (CH), 128.33 (CH), 128.47 (CH), 128.58 (CH), 128.71 (CH), 128.76 (CH), 135.75 (C), 136.40 (C), 136.61 (C), 136.86 (C), 137.42 (C3), 148.19 (C3'), 151.06 (C4'), 156.27 (C2), 156.64 (C8a), 162.03 (C5), 164.42 (C7), 178.78 (C=O) ppm. ESI m/z (%): 685.26 (M^++Na, 47%), 453.28 (100%).
The NMR spectroscopy data are similar to those found in the literature by Kajjout et al., 2002 and Kajjout & Rolando, 2011.

4.4.1.4 Experiment 14 (Section 4.1, *Scheme 4*)

To a solution of quercetin **1** (0.200 g, 0.660 mmol) in DMSO (3 mL), potassium carbonate (0.411 g, 2.97 mmol, 4.5 equiv.) and BnBr (0.355 mL, 2.97 mmol, 4.5 equiv.) were added under argon. The reaction mixture was stirred at room temperature for 14 h. The resulting mixture was diluted with iced water (50 mL), extracted with ethyl acetate (3 x 50 mL). Then, the organic layer was washed with water (3 x 80 mL), dried ($MgSO_4$) and evaporated, affording a complex mixture that was submitted to flash column chromatography (silica gel, dichloromethane). The crude residue was purified by crystallization from dichloromethane-hexane, affording a compound **2** as a yellow solid, 0.257 g, in 35.1% yield (***Scheme 4***, **Section 4.1**). MP 147-149 ºC [150-152 ºC - Bouktaib et al., 2002; 149-150 ºC -Z-H Shi et al., 2012]. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.08 (s, 2H, 3-OCH_2Ph), 5.14 (s, 2H, 7-OCH_2Ph), 5.20 (s, 2H, 4'-OCH_2Ph), 6.45 (d, *J* = 2.0 Hz, 1H, H-6), 6.51 (d, *J* = 2.4 Hz, 1H, H-8), 6.96 (d, *J* = 9.2 Hz, 1H, H-5'), 7.28-7.30 (m, 3H + OH), 7.35-7.47 (m, 13H, aromatic H), 7.6305 (d, *J* = 2.0 Hz, 1H, H-2'), 7.631 (dd, *J* = 9.2 and 2.0 Hz, 1H, H-6') ppm. ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 70.40 (CH_2), 71.10 (CH_2), 74.22 (CH_2), 92.98 (C8), 98.63 (C6), 106.18 (C5a), 111.53 (C5'), 114.86 (C6'), 121.86 (C2'), 123.92 (C2), 127.44 (CH), 127.85(CH), 128.17 (CH), 128.25 (CH), 128.32 (CH), 128.65 (CH), 128.72 (CH), 128.77 (CH), 128.82 (CH), 135.63 (C), 135.77 (C),

136.40 (C), 137.58 (C), 145.55 (C3'), 147.81(C4'), 156.31 (C), 156.71 (C8a), 161.99 (C5), 164.44 (C7), 178.81 (C=O) ppm.
ESI m/z (%): 595.20 (M^++Na^+, 13%), 611.17 (100%), 453.27 (30%), 368.59 (33%).
The NMR spectroscopy data are similar to those found in the literature by Kajjout et al., 2002 and Shi et al., 2012.

4.4.1.5 Experiment 15 (Section 4.1, *Scheme 5*)

Step 1: The four free hydroxyl groups of rutin **4** were benzylated using an excess of benzyl bromide (8 equiv.) and potassium carbonate (8 equiv.) in DMF, during 16 h at room temperature. The resulting mixture was diluted with ethyl acetate (100 mL) and washed with water (3 x 75 mL). The organic layer was dried ($MgSO_4$) and evaporated. The residue was obtained as a beige syrup (1.046g, 93.6%) that was identified as **5 (Step 1, *Scheme 5*, Section 4.1)**. ^{1}H NMR (400 MHz, DMSO-D_6) *inter alia* 5.14, 5.17, 5.24 and 5.25 ($PhCh_2O$) ppm. **Step 2:** The hydrolysis of the glycosidic bond of the *O*-tetrabenzylated rutin **5** was performed using a mixture of MeOH/HCl (98:2) at reflux (65 ºC) which led to the desired product 5,7-bis(benzyloxy)-2-[3',4'-bis(benzyloxy)phenyl]-3-hydrixy-4*H*-chromen-4-one (**6)** and as a light yellow powder (210 mg, 46.1% yield), (***Scheme 5*, Section 4.1**). This NMR spectroscopy data are similar to those found in the literature to Kajjout et al., 2011. MP: 169-171 ºC. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.12 (s, 2H, 7-OCH_2Ph), 5.22 (s, 2H, 5-OCH_2Ph) 5.24 (s, 2H, 4'-OCH_2Ph), 5.26 (s, 2H, 3'-OCH_2Ph), 6.47 (d, *J* = 2.0 Hz, 1H, H-6), 6.58 (d, *J* = 2.0 Hz, 1H, H-8), 7.03 (d, *J* = 8.8 Hz, 1H, H-5'), 7.32 -7.62 (m, 20H, 4 x Ph), 7.77 (dd, *J* = 8.8 and 2.0 Hz, 1H, H-6'), 7.90 (d, *J* = 2.4 Hz, 1H, H-2') ppm.$^{(*)}$ ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 70.52 (7-OCH_2Ph), 70.64 (5-OCH_2Ph), 70.89 (4'-OCH_2Ph), 71.48 (3'-OCH_2Ph), 93.62 (C8), 97.51 (C6), 106.62 (C5a), 114.14 (C2'), 121.22 (C6'), 124.19 (C2), 126.59 (CH), 127.15 (CH), 127.50 (CH), 127.62 (CH), 127.75 (CH), 127.84 (CH), 127.87 (CH), 128.45 (CH), 128.52 (CH), 128.62 (CH), 128.74 (CH), 135.55 (CH), 136.12(C), 136.79 (C), 137.10 (C), 137.63 (C4'), 141.98 (C3), 148.53 (C1'), 150.14 (C3'), 158.60 (C8a), 159.29 (C5), 163.20 (C7), 171.64 (C=O) ppm. ESI m/z (%): 685.34 (M^++Na, 59.34%), 663.31 (49.2%), 368.58 (100%). $^{(*)}$ the proton of the OH was not detected in the proton ^{1}H-NMR spectrum.

4.4.2 Methylation reaction

4.4.2.1 Experiment 16 (Section 4.2, *Scheme 6*)

2-(3'4'-dimethoxyphenyl)-5-hydroxy-3,7-dimethoxy-4H-chromen-4-one: A mixture of quercetin **1** (0.200 g, 0.660 mmol), dimethyl sulphate (0.124 µL, 1.32 mmol), potassium carbonate (0.366 g, 2.65 mmol) in acetone (30 mL) was stirred at 45-50 ºC for 18 h, to afford the *O*-tetramethylated quercetin **12**. The solvent was removed under vacuum. The residue was suspended in ammonium solution (70:10 v/v) and extracted with ethyl acetate (3 x 50 mL). The organic layer was dried ($MgSO_4$), evaporated and the residue obtained was a yellow solid that was identified as **12**, 116 mg in 49% yield. MP: 95-97 ºC. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 3.88 (s, 3H, OMe), 3.89 (s, 3H, OMe), 3.978 (s, 3H, OMe), 3.984 (s, 3H, OMe), 6.38 (d, *J* = 2.4 Hz, 1H, H-6), 6.46 (d, *J* = 2.4 Hz, 1H, H-8), 7.00 (d, *J* = 8.4 Hz, 1H, H-5´), 7.70 (d, *J* = 2.4 Hz, 1H, H-2´), 7.75 (dd, *J*= 8.4, 2.0 Hz, 1H, H-6´), 12.62 (1H, broad s, OH) ppm. ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 55.73 (OCH_3), 55.90 (OCH_3), 55.97 (OCH_3), 60.08 (OCH_3), 92.09 (C8), 97.74 (C6), 105.91 (C5a), 110.77 (C5'), 111.16 (C2'), 122.09 (C6'), 122.80 (C1'), 138.86 (C3), 148.67 (C3'), 151.29 (C4'), 155.71 (C4), 156.60 (C8a), 161.89 (C5), 165.35 (C7), 176.62 (C=O) ppm.

4.4.3 Glucuronidation reactions

4.4.3.1 Experiments 17,18 and 19 (Section 4.3, *Scheme 7, 8* and *9*)

O-tribenzylated **2** (Experiment 17) and *O*-tetrabenzylated **3** (Experiment 18) derivatives of quercetin were reacted with potassium carbonate (2 equiv.) and glucuronic acid derivative **8** (2 equiv.) in DMF, at room temperature, under argon. The mixtures were stirred for 24 h. Each reaction mixture was diluted with EtOAc (30 mL) and was washed with water (3 x 30 mL), dried ($MgSO_4$) and evaporated, affording a complex mixture that was submitted to flash column chromatography (silica gel; dichloromethane:ethyl acetate, 85:15) but the compounds formed were not possible to be separated,: (***Scheme 7*** and ***8***, **Section 4.3**). The residue obtained from experiment 17 showed in the ^{1}H-NMR spectrum several compounds including starting materials. In experiment 18, also a very complex mixture was obtained in the ^{1}H-NMR spectrum of the residue, and the fractions collected after column chromatography were not possible to identify.

A mixture of *O*-tetrabenzylated quercetin **6** (experiment 19) (0.200 g, 0.300 mmol), potassium carbonate (0.084 g, 0.600 mmol), acetobromo-α-D-glucuronic acid methyl ester **8** (0.240 g, 0.600 mmol) were dissolved in DMF (3 mL) under argon and the solution was stirred at room temperature for 20 h. The reaction mixture was diluted with ethyl acetate (50 mL) and washed with water (3 x 30 mL). The organic layer was dried ($MgSO_4$) and the solvent evaporated. The ^{1}H-NMR spectrum of the crude residue showed a complex mixture of compounds.

In all the experiments 17, 18 and 19, only a small amount of the β-anomer expected **9**, **10** and **11,** seemed to be formed based on the presence of a doublet at 5.78 ppm, J = 8.0 Hz, that appears in the corresponding ^{1}H-NMR spectrum mixtures.

4.4.3.2 Experiment 20 (Section 4.3, *Scheme 10*)

A mixture of the *O*-tetramethylated quercetin **7** (0.100 g, 0.280 mmol), potassium carbonate (0.076 g, 0.550 mmol) and acetobromo-α-D- glucuronic acid methyl ester **8** (0.220 g, 0.550 mmol) was dissolved in DMF (3 mL) under argon and the solution was stirred for 20 h at room temperature (RT). The reaction mixture was diluted with ethyl acetate (50 mL) and washed with water (3 x 30 mL). The organic layer was dried ($MgSO_4$) and the solvent evaporated. The residue obtained gave a very complex mixture in ^{1}H-NMR and was submitted to a column chromatography using silica gel 60A (60-200 μm) and a mixture of dichloromethane/ethyl acetate (85:15) was used as eluent, but the products were not possible to be separated. In this experiment the β-anomer of compound 13 seemed to be formed in a small amount, based on the presence of doublet at 5.77 ppm, J = 7.6 Hz, that appears in the corresponding ^{1}H-NMR spectrum mixture.

Chapter 5.

Conclusions and Future Perspectives

Chapter 5. Conclusions and Future perspectives

The nutritional characterization of Roman chamomile (*C. nobile*) was performed and carbohydrates were the most abundant macronutrients, followed by proteins. The sugars fructose, glucose, sucrose and trehalose were identified and quantified in this wild chamomile, being fructose the predominant sugar found. In lipidic fraction the predominant compounds were linoleic and oleic acids, and polyunsaturated fatty acids (PUFA) predominated over saturated fatty acids (SFA). Regarding tocopherols, only α- and γ- tocopherols were found in this wild chamomile. Different types of preparations, infusion, decoction and hydroalcoholic extract of this wild chamomile were evaluated for the antioxidant activity and submitted to a detailed analysis of phytochemicals (organic acids and phenolic compounds). Oxalic, quinic, malic, citric and fumaric acids were quantified in the three preparations of *C. nobile*. Malic acid was the most abundant organic acid in the three preparations, and infusion showed the highest content in organic acids. The phenolic profile of the different preparations was identical, varying only in the concentrations found. Thirty different phenolic compounds were detected in the three preparations, including phenolic acids (hydroxycinnamic derivatives mainly, caffeoylquinic acids), flavonols (quercetin, kaempferol and myricetin derivatives) and flavones (apigenin and luteolin derivatives). The most abundant compounds in infusion and hydroalcoholic extract were 5-*O*-caffeoylquinic acid and an apigenin derivative. A degradation of the compounds was observed in both infusion and decoction preparations, mainly in the last one. This could be related with the higher thermical impact applied to obtain this type of preparation, when compared to the infusion. The hydroalcoholic extract gave the highest antioxidant activity in the β-carotene bleaching activity and TBARS inhibition assays (which can be related to its higher content in phenolic compounds). The highest radical scavenging activity and reducing power were observed in the infusion. Otherwise, decoctions presented the lowest antioxidant potential (probably due to its lower content in phenolic compounds and organic acids). Antitumor properties of *C. nobile* hydroalcoholic extract, decoction and infusion were evaluated on five human tumor cell lines (MCF-7, NCI-H460, HCT-15, HeLa and HepG2). The hydroalcoholic extract presented the highest growth inhibitory activity for all the cell lines, and decoction preparation had no antitumor effects at the maximal concentration used.

The antiangiogenic activity of *C. nobile* hydroalcoholic extract and infusion, and its main phenolic compounds: apigenin, apigenin-7-*O*-glucoside, caffeic acid, chlorogenic acid, luteolin, luteolin-7-*O*-glucoside were evaluated for their ability to interact with the VEGFR-2 tyrosine kinase domain, using an enzymatic assay. The hydroalcoholic extract showed higher phosphorylation inhibition than the infusion, what is in agreement with the higher phenolic compounds amount, antioxidant and antitumor activities also previously reported for hydroalcoholic extract. Regarding phenolic compounds, luteolin and apigenin were the most potent in inhibiting VEGFR-2 phosphorylation, leading us to believe that these compounds are involved in the antiangiogenic activity revealed by the hydroalcoholic extract.

The antioxidant properties of German chamomile (*M. recutita*) infusion and decoction were similar, and showed better results than the hydroalcoholic extract in the DPPH and β-carotene bleaching inhibition assays. The hydroalcoholic extract of this wild chamomile gave the best results for reducing power and TBARS assays. Antitumor effects of *M. recutita* infusion and hydroalcoholic extract showed to be selective only for HCT-15 and HeLa cell lines, but none of the three preparations showed hepatotoxicity for PLP2 cells. Once again, decoction had no antitumor activity at the maximal concentration used. Phenolic acids and derivatives, and flavonoids such as flavonols and flavones were found in *M. recutita*. Twenty phenolic compounds were detected. Luteolin-*O*-acylhexoside was the most abundant compound identified in the three preparations and the hydroalcoholic extract was the preparation that presented the highest quantity of this compound. Protocatechuic acid, *cis* 5-*O*-caffeolyquinic acid, 5-*O*-feruloyquinic acid and 4,5-*O*-dicaffeoylquinic acid were only detected in infusion and decoction, and myricetin-*O*-hexoside, quercetin 7-*O*-acetylhexoside and 1,5-dicaffeolyquinic acid were only found in the hydroalcoholic extract.

Wild fruits of *Arbutus unedo*, *Prunus spinosa*, *Rosa canina* and *Rosa micrantha* are rich in a variety of phenolic compounds, and the present study represents a contribution to the phenolic characterisation of these wild fruits with acknowledged antioxidant activity and traditionally used for several folk medicinal applications. These compounds have potent antioxidant properties, which have been related to anticancer activity. *P. spinosa* fruits were the only ones that presented phenolic acids, all belonging to the hydroxycinnamic acid derivative sub group. The major phenolic compound found in this wild fruit was identified as 3-*O*-caffeoylquinic acid. In all the studied samples of the wild fruits, quercetin 3-*O*-rutinoside and quercetin 3-*O*-glucoside were found. Quercetin pentoside

and quercetin rhamnoside were also found in all samples. Quercetin hexoside was only found in *P. spinosa*, *R. canina* and *R. micrantha.* Other detected flavonols, corresponding to kaempferol, isorhamnetin and myricetin derivatives were found in these fruits. Two myricetin rhamnosides were found in *A. unedo*. Taxifolin (flavanonol) and eriodictyol (flavanone) derivatives were identified in *R. canina* and *R. micrantha* wild fruits. *P. spinosa* was the fruit that presented the highest concentration in phenolic acids. *A. unedo* fruits presented quercetin 3-*O*-glucoside as the main flavonol. In *R. canina* and *R. micrantha* wild fruits taxifolin pentoside was the major flavananol derivative quantified, and *R. micrantha* presented the highest amount of total flavonoids. Catechins and proanthocyanidins (flavan-3-ols) were other relevant class of flavonoids found in *A.unedo*, *R. canina* and *R. micrantha* fruits. (+)-Catechin was the most abundant flavan-3-ol found in *A. unedo* and *R. canina*. It was also a prominent compound in *R. micrantha*, besides a glycosylated proanthocyanidin dimer. *A. unedo* presented the highest concentration in proanthocyanidins. Flavan-3-ols were not detected in *P. spinosa*, and *A. unedo* was the only wild fruit studied that presented galloyl derivatives. Proanthocyanidin dimer monoglycoside was the most abundant flavan-3-ol present in *R. canina* and *R. micrantha* fruits. The anthocyanin profile obtained for *P. spinosa*, was more complex that those found in other fruits. For *R. canina* and *R. micrantha* only one anthocyanin was detected, namely cyanidin-3-*O*-glucoside. Three differents anthocyanins were identified in *A. unedo*, cyanidin 3-*O*-glucoside, cyanidin 3-*O*-pentoside and delphinidin-3-*O*-glucoside, but the most abundant was cyanidin 3-*O*-glucoside. Cyanidin 3-*O*-glucoside was found in all the samples, being the major anthocyanin in most of them, with exception of *P. spinosa* in which cyanidin 3-*O*-rutinoside and peonidin 3-*O*-rutinoside were the main anthocyanins. The highest phenolic acids, flavonols and anthocyanins contents were found in *P. spinosa*, despite no flavan-3-ols were detected in this sample. The comparative study of the bioactivity of different enriched phenolic extracts of wild fruits (PE- non-anthocyanin phenolic compounds enriched extract) gave higher antioxidant properties than the corresponding AE (anthocyanins enriched extracts), and in accordance with their chemical characterization. *A. unedo* presented the highest antioxidant activity in all the preformed assays. The second one with the highest antioxidant effects was *P. spinosa*, while *Rosa* species revealed the lowest antioxidant activity. The antitumor potential of all fruit extracts were tested in breast, lung, colon, cervical and hepatocellular cell lines. Hepatotoxicity was evaluated using a porcine liver primary cell culture. All the extracts inhibited the growth of tumor cell lines, except *R. canina* PE and AE, *P. spinosa*

AE and *R. micrantha* AE for the breast carcinoma (MCF-7). *A. unedo*, followed by *P. spinosa* PE gave the best antitumor potential. None of the samples showed toxicity for non-tumor liver primary cell culture. Regarding the chemical characterization of the metionated samples, the presence of galloyl derivatives exclusively in *A. unedo* wild fruits could be related to its higher bioactivity. The studied fruits may have great potential for food industries as a source of colours and flavours. The bioactivity of the studied wild fruits proved to be more related with the phenolic compounds profile than with the amounts present in each extract, and could be considered in the design of new formulations of dietary supplements or functional foods.

The chemical synthesis of metabolites/derivatives of quercetin proved to be quite difficult. The protection of the hydroxy groups present in quercetin was done following the literature, using the protection model described by Bouktaib et al (2002) and Kajjout & Rolando (2011). Both authors used benzyl as a protecting group because it is easily introduced by reaction with a benzyl halide and also easily removed by hydrogenolysis. Nevertheless, we have used different reaction conditions, because we were unable to reproduce the literature results. Thus, several benzylated derivatives of quercetin were obtained, but their separation by chromatographic column was not possible using the mixtures of eluents suggested by both research groups, probably due to the formation of more benzylated derivatives in our reactions. From two of our benzylation attemps it was possible to obtain the 3,7,3',4'-*O*-tetrabenzylated derivative in one case, and the 3,7,3´-*O*-tribenzylated derivative in other case, which were identified by ^{1}H and ^{13}C NMR and comparing with the spectroscopic data of the literature. The 5,7,3',4'-*O*-tetrabenzylated quercetin was obtained from benzylation of rutin (a 3-*O*-disaccharide of quercetin) followed by 3-*O*-deglycosylation. The glucuronidation of *O*-tri- and of the two *O*-tetrabenzylated quercetin derivatives was attempted by reacting them with acetobromo-α-D-glucuronic acid methyl ester, giving mixtures of various compounds including a small amount of the expected glucurodinated β-anomers.

In face of the results obtained in the reactions involving benzyl protection followed by glucuronidation, it was decided to investigate the use of methylation, to protect the hydroxyls with a less bulky group, the methoxylated compounds are also known as flavonoids metabolites. The 3,7,3'4'-*O*-tetramethylated quercetin was obtained according to the reactivity described in the literature by Shi et al. 2012 and it was characterized by melting point (Mp), ^{1}H and ^{13}C NMR. As far as our knowledge it is not described in the literature. The glucuronidation of this tetramethylated quercetin did not work. The crude

residue revealed that a very complex mixture was formed, and it was not possible to obtain the expected 5-*O*-glucuronide-3,7,3',4'-*O*-tetramethylated quercetin.

Further studies, are needed in the glucuronidation reaction of flavonoids to avoid the formation of complex mixtures that prevented us to get the glucuronide derivatives wanted, in higher amounts and in a pure form..

Chapter 6.
References

Chapter 6. References

Abreu, R. M. V., Ferreira, I. C. F. R., Calhelha, R. C., Lima, R. T., Vasconcelos, M. H., Adega, F., Chaves, R., Queiroz, M. J. R. P. **(2011)**. Anti-hepatocellular carcinoma activity using human HepG2 cells and hepatotoxicity of 6-substituted methyl 3-aminothieno[3,2-*b*]pyridine-2-carboxylate derivatives: *In vitro* evaluation, cell cycle analysis and QSAR studies. European Journal of Medicinal Chemistry, 46, 5800-5806.

Abreu, R. M., Froufe, H. J., Queiroz, M. J. R. P., Ferreira, I. C. F. R. **(2010)**. MOLA: a bootable, self-configuring system for virtual screening using AutoDock4/Vina on computer clusters. Journal of cheminformatics, 2, 10.

Ader, P., Wessmann, A., Wolffram, S. **(2000)**. Bioavailability and metabolism of the flavonol quercetin in the pig. Free radical biology and medicine, 28, 1056-1067.

Adlercreutz, H., Honjo, H., Higashi, A., Fotsis, T., Hamalainen, E., Hasegawa, T., Okada H. **(1991)**. Urinary excretion of lignans and isoflavonoid phytoestrogens in Japanese men and women consuming a traditional Japanese diet. The American Journal of Clinical Nutrition, 54, 1093-1100.

Alvesalo, J., Vuorela, H., Tammela, P., Leinonen, M., Saikku, P., Vuorela, P. **(2006).** Inhibitory effect of dietary phenolic compounds on Chlamydia pneumoniae in cell cultures. Biochemical Pharmacology, 71, 735-741.

Anderson, R. A., Broadhurst, C. L., Polansky, M. M., Schmidt, W. F., Khan, A., Flanagan, V. P., Schoene, N. W., Graves, D. J. **(2004)**. Isolation and characterization of polyphenol type-A polymersfrom cinnamon with insulin-like biological activity. Journal of Agricultural and Food Chemistry, 52, 65-70.

AOAC. **(1995)**. Official methods of analysis (16th Ed.). Arlington VA, USA: Association of Official Analytical Chemists.

Arinç, E., Ylmaz, D., Bozcaarmutlu, A. **(2015)**. Mechanism of inhibition CYP1A1 and glutathione s-transferase activities in fish liver by quercetin, resveratrol, naringenin, hesperidin, and rutin. Nutrition and cancer, 67, 137-144.

Barros, L., Alves, C. T., Dueñas, M., Silva, S., Oliveira, R., Carvalho, A. M., Henriques, M., Santos-Buelga, C., Ferreira, I.C.F.R. **(2013)**. Towards antifungal compounds exploring phenolic extracts of wild medicinal flowers from Portugal. Industrial Crops and Products, 44, 104-110.

Barros, L., Alves, C. T., Dueñas, M., Silva, S., Oliveira, R., Carvalho, A. M., Henriques, M., Santos-Buelga, C., Ferreira, I. C. F. R. **(2013)**. Characterization of phenolic compounds in wild medicinal flowers from Portugal by HPLC-DAD-ESI/MS and evaluation of antifungal properties. Industrial Crops and Products, 44, 104-110.

Barros, L., Carvalho, A. M., Ferreira, I. C. F. R. **(2011)**. Exotic fruits as a source of important phytochemicals: Improving the traditional use of *Rosa canina* fruits in Portugal. Food Reseach International, 44, 2233-2236.

Barros, L., Carvalho, A. M., Morais, J. S., Ferreira, I. C. F. R. **(2010)**. Strawberry-tree, blackthorn and rose fruits: Detailed characterization in nutrients and phytochemicals with antioxidant properties. Food Chemistry, 120, 247-254.

Barros, L., Carvalho, A. M., Morais, J. S., Ferreira, I. C. F. R. **(2010)**. Strawberry-tree, blackthorn and rose fruits: Detailed characterization in nutrients and phytochemicals with antioxidant properties. Food Chemistry, 1, 247-254.

Barros, L., Dueñas, M., Dias, M. I., Sousa, M. J., Santos-Buelga, C., Ferreira, I. C. F. R. **(2012)**. Phenolic profiles of *in vivo* and *in vitro* grown *Coriandrum sativum* L. Food Chemistry, 132, 841-848.

Barros, L., Oliveira, S., Carvalho, A. M., Ferreira, I. C. F. R. **(2010)**. *In vitro* antioxidant properties and characterization in nutrients and phytochemicals of six medicinal plants from the Portuguese folk medicine. Industrial Crops and Products, 32, 572-579.

Barros, L., Pereira, C., Ferreira, I. C. F. R. **(2012)**. Optimized analysis of organic acids in edible mushrooms from Portugal by ultra fast liquid chromatography and photodiode array detection. Food Analytical Methods, in press, DOI: 10.1007/s12161-012-9443-1

Borkowski, B., Lutomski, J., Skrzydlewska, E., Zygmunt, B. **(1994)**. Rosliny lecznicze w fitoterapii. IRiPZ Poznan, 470-471.

Bouktaib, M., Atmani, A., Rolando, C. **(2002)**. Regio- and stereoselective synthesis of the major metabolite of quercetin, quercetin-3-*O*-β-D-glucuronide. Tetrahedron Letters, 43, 6263-6266.

Bravo, L. **(1998)**. Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. Nutrition Reviews, 56, 317-333.

Brusselmans, K., Vrolix, R., Verhoeven, G., Swinnem, J. V. **(2005)**. Induction of cancer cell apoptosis by flavonoids is associated with their ability to inhibit fatty acid synthase activity. Journal of Biological Chemistry, 280, 5636-5645.

Cai, X., Ye, T., Liu, C., Lu, W., Lu, M., Zhang, J., Wang, M., & Cao, P. **(2011)**. Luteolin induced G2 phase cell cycle arrest and apoptosis on non-small cell lung cancer cells. Toxicology *in Vitro*, 25, 1385-1391.

Camejo-Rodrigues, J. S. **(2006)**. *Recolha dos 'Saber-Fazer' Tradicionais das Plantas Aromáticas e Medicinais, Concelhos de Aljezur, Lagos e Vila do Bispo*. Associação Aflosul, Bordeira.

Camejo-Rodrigues, J., Ascensão, L., Bonet, M. A., Vallès, J. **(2003)**. Na ethnobotanical study of medicinal and aromatic plants in the Natural Park of "Serra de São Mamede" (Portugal. Journal of Ethnopharmacology, 89, 199-209.

Canivenc-Lavier, M. C., Vernevaut, M. F., Totis, M., Siess, M. H., Magdalou, J., Suschetet, M. **(1996)**. Comparative effects of flavonoids and model inducers on drug-metabolizing enzymes in rat liver. Toxicology, 114, 19–27.

Carnat, A., Carnat, A. P., Fraisse, D., Ricoux, L., Lamaison J. L. **(2004)**. The aromatic and polyphenolic composition of Roman camomile tea. Fitoterapia, 75, 32–38.

Carvalho, A. M. **(2010)**. *Plantas y sabiduría popular del Parque Natural de Montesinho. Un estudio etnobotánico en Portugal*. Biblioteca de Ciencias 35. Madrid: Consejo Superior de Investigaciones Científicas.

Castroviejo, S., Aedo, C., Cirujano, S., Laínz, M., Montserrat, P., Morales, R., Muñoz Garmendia, F., Navarro, C., Paiva, J., Soriano, C. (eds.) **(2001).** Flora iberica 6. Real Jardín Botánico, CSIC, Madrid.

Chao, C. L., Hou, Y. C., Lee Chao, P. D. L., Weng, C. S., Ho, F. M. **(2009)**. The antioxidant effects of quercetin metabolites on the prevention of high glucose-induced apoptosis of human umbilical vein endothelial cells. British Journal of Nutrition, 101, 1165-1170.

Choi, J. A., Kim, J. Y., Lee, J. Y., Kang, C. M., Kwon, H. J., Yoo, Y. D., Kim, T. W., Lee, Y. S., Lee, S. J. **(2001)**. Induction of cell cycle arrest and apoptosis in human breast cancer cells by quercetin. International Journal of Oncology, 19, 837–844.

Clifford, M. N., Johnston, K. L., Knight, S., Kuhnert, N. A. **(2003)**. A hierarchical scheme for LC-MSn identification of chlorogenic acids. Journal of Agricultural and Food Chemistry, 51, 2900-2911.

Clifford, M. N., Knight, S., Kuhnert, N. A. **(2005)**. Discriminating between the six isomers of dicaffeoylquinic acid by LC-MSn. Journal of Agricultural and Food Chemistry, 53, 3821-3832.

Clifford, M. N., Wu, W., Kirkpatrick, J., Kuhnert, N. A. **(2007)**. Profiling the chlorogenic acids and other caffeic acid derivatives of herbal chrysanthemum by HPLC-MSn. Journal of Agricultural and Food Chemistry, 55, 929-936.

Cody, V. **(1988)**. Crystal and molecular structure of flavonoids. In Plant flavonoids in biology and medicine II: Biochemical, cellular and medicinal properties, 29-44.

Cos, P., Ying, L., Calomme, M., Hu, J. P., Cimanga, K., Van Poel, B., Pieters, L., Vlietnck, A. J., Vanden Berghe, D. **(1998)**. Structure-activity relationship and classification of flavonoids as inhibitors of xanthine oxidase and superoxide scavengers. Journal of Natural Products, 61, 71-76.

Coughtrie, M. H., Sharp, S., Maxwell, K., Innes, N. P. **(1998)**. Biology and function of the reversible sulfation pathway catalysed by human sulfotransferases and sulfatases. Chemico-Biological Interactions, 109, 3-27.

Crevin, J. K., Philpott, J. **(1990)**. Herbal medicine past and present. Vol. II Duke University Press.

Cushine, T. P. T., Lamb, A. J. **(2005)**. Antimicrobial activity of flavonoids. International Journal of Antimicrobial agents, 26, 343-356.

Day, A. J., Canada, F. J., Diaz, J. C., Kroon, P. A., Mclauchlan, W. R., Faulds, C. B., Plumb, G. W., Morgan, M. R., Williamson, G. **(2000)**. Dietary flavonoid and isoflavone glycosides are hydrolysed by the lactase site of lactase phlorizin hydrolase. FEBS Letters, 468, 166-70.

Day, A. J., Mellon, F., Barron, D., Sarrazin, G., Morgan, M. R., Williamson, G. **(2001)**. Human metabolism of dietary flavonoids: identification of plasma metabolites of quercetin. Free Radical Research, 35, 941-952.

Day, A. J., Williamson, G. **(2001)**. Biomarkers for exposure to dietary flavonoids: a review of the current evidence for identification of quercetin glycosides in plasma. British Journal of Nutrition, 86 (1Suppl), S105–110.

Delgado, G. **(1992)**. Organic natural products from some Mexican plants. Trends in Organic Chemistry, 3, 129-140.

Dixon, R. A., Pasinetti, G. M. **(2010)**. Flavonoids and isoflavonoids: from plant biology to agriculture and neuroscience. Plant Physiology, 154, 453-457.

Dixon, R. A., Steele, C. L. **(1999)**. Flavonoids and isoflavonoids – a gold mine for metabolic engineering. Trends in plant science, 4, 394-400.

Dixon, R.A., Xie, D.-Y, Sharma, S. B. **(2005)**. Proanthocyanidins – a final frontier in flavonoid research? New Phytologist, 165, 9-28.

Donovan, J. L., Manach, C., Faulks, R. M., Kroon, P. A. **(2006)**. Absorption and metabolism of plant secondary metabolites. In: Crozier A, Clifford MN, Ashihara H, editors. Plant Secondary Metabolites: Occurrence, Structure and Role in the Human Diet. Blackwell Publishing; Oxford, UK, pp. 303–351.

Dueñas, M., Chornet, H. M., Pérez-Alonso, J. J., Paola-Naranjo, R. D., González-Paramás, A. M., Santos-Buelga, C. **(2008)**. Preparation of quercetin glucuronides and characterization by HPLC–DAD–ESI/MS. European Food Research and Technology, 227, 1069–1076.

Dugo, P., Mondello, L., Errante, G., Zappia, G., Dugo, G. **(2001)**. Identification of anthocyanins in berries by narrow-bore high performance liquid chromatography with electrospray ionization detection. Journal of Agricultural and Food Chemistry, 49, 3987–3992.

Elliotti, A. J., Scheiber, S. A., Thomas, C., Pardini, R. S. **(1992)**. Inhibition of glutathione reductase by flavonoids. Biochemical Pharmacology, 44, 1603-1608.

Erlund, I., Alfthan, G., Maenpaa, J., Aro, A. **(2001)**. Tea and coronary heart disease: the flavonoid quercetin is more bioavailable from rutin in women then in men. Archieves. Journal *of* Clinical Nutrition, 161, 1919-1920.

Evans, S., Dizeyi, N., Abrahamsson, P. A., Persson, J. **(2009)**. The effect of a novel botanical agent TBS-101 on invasive prostate cancer in animal models. Anticancer Research, 10, 3917-3924.

Fecka, I. **(2009)**. Qualitative and quantitative determination of hydrolysable tannins and other polyphenols in herbal products from meadowsweet and dog rose. Phytochemical Analysis, 20, 177-190.

Ferguson, P. J., Kurowska, E., Freeman, D. J., Chambers, A. F., Koropatnick, D. J. **(2004)**. A flavonoid fraction from cranberry extract inhibits proliferation of human tumor cell lines. Journal of Nutrition, 134, 1529-1535.

Ferrali, M., Signorini, C., Caciotti, B., Sugherini, L., Ciccoli, L., Giachetti, D., Comporti, M. **(1997)**. Protection against oxidative damage of erythrocyte membranes by the flavonoid quercetin and its relation to iron chelating activity. FEBS letters, 416, 123-129.

Ferreres, F., Silva, B. M., Andrade, P. B., Seabra, R. M., Ferreira, M. A. **(2003)**. Approach to the study of C-glycosyl flavones by ion trap HPLC-PAD-ESI/MS/MS: application to seeds of quince (*Cydonia oblonga*). Phytochemical Analysis, 14, 352-359.

Forkmann, G., Martens, S. **(2001)**. Metabolic engineering and applications of flavonoids. Current Opinion in Biotechnology, 12, 155-160.

Fortalezas, S., Tavares, L., Pimpão, R., Tyagi, M., Pontes, V., Alves, P. M., McDougall, G., Stewart, D., Ferreira, R. B., Santos, C. N. **(2010)**. Antioxidant properties and neuroprotective capacity of strawberry tree fruits (*Arbutus unedo*). Nutrients 2, 214-229

Franke, A., Custer, L. J., Cerna, C. M., Narala, K. K. **(1994)**. Quantitation of phytoestrogens in legumes by HPLC. Journal of agricultural and food chemistry, 42, 1905-1943.

Fujii, T., Ikeda, K., Saito, M. **(2011)**. Inhibitory effect of rose hip (*Rosa canina* L.) on melanogenesis in mouse melanoma cells and on pigmentation in brown guinea pigs. Bioscience Biotechnology and Biochemistry, 75, 489-495.

Galati, G., O'Brien, P. J. **(2004)**. Potential toxicity of flavonoids and other dietary phenolics: significance for their chemopreventive and anticancer properties. BioFactors, 37, 287-303.

Ganhão, R., Estévez, M., Kylli, P., Heinonen, M., Morcuende, D. **(2010)**. Characterization of selected wild mediterranean fruits and comparative efficacy as inhibitors of oxidative reactions in emulsified raw pork burger patties. Journal of Agriculture and Food Chemical, 58, 8854-8861.

García-Marino, M., Hernández-Hierro, J. M., Rivas-Gonzalo, J. C., Escribano-Bailón, M. T. **(2010)**. Colour and pigment composition of red wines obtained from co-maceration of Tempranillo and Graciano varieties. Analytical Chimica Acta, 660, 134-142.

Gómez, D. Villar, L. **(2004).** Arbutus L. In Castroviejo, S., Aedo, C., Laínz, M., Muñoz Garmendia, F., Nieto Feliner, G., Paiva, J. & Benedí, C. (eds.). Flora iberica 4: 514-516. Real Jardín Botánico, CSIC, Madrid.**Graefe**, E. U., Derendorf, H., Veit, M. **(1999)**. Pharmacokinetics and bioavailability of the flavonol quercetin in humans. International journal of clinical pharmacology and therapy, 37, 219-233.

Gopalan, V., Pastuzyn, A., Galey, W. R., Glew, R. H. **(1992)**. Exolytic hydrolysis of toxic plant glucosides by guinea pig liver cytosolic β-glucosidase. Journal of Biological Chemistry, 267, 14027-14032.

Graefe, E. U., Derendorf, H., Veit, M. **(1999)**. Pharmacokinetics and bioavailability of the flavonol quercetin in humans. International journal of clinical pharmacology and therapy, 37, 219-233.

Graefe, E. U., Wittig, J., Mueller, S., Riethling, A. K., Uehleke, B., Drewelow, B., Pforte, H., Jacobasch, G., Derendorf, H., Veit, M. **(2001)**. Pharmacokinetics and bioavailability of quercetin glycosides in humans. Journal of clinical pharmacology, 41, 492-499.

Granzera, M., Schneider, P., Stuppner, H. **(2006)**. Inhibitory effects of the essential oil of chamomile (*Matricaria recutita*) and its major constituents on human cytochrome P450 enzymes. Life Sciences, 78, 856-861.

Griffiths, L. A., Barrow, A. **(1972)**. Metabolism of flavonoids compounds in germ-free rats. Biochemical Journal, 130, 1161-1162.

Guil, J. L., Torija, M. E., Giménez, J. J., Rodriguez, I. **(1996)**. Identification of fatty acids in edible wild plants by gas chromatography. Journal of Chromatography A, 719, 229-235.

Guimarães, R., Barros, L., Carvalho, A. M., Ferreira, I. C. F. R. **(2010)**. Studies on chemical constituents and bioactivity of *Rosa micrantha*: an alternative antioxidants source for food, pharmaceutical, or cosmetic applications. Journal of Agricultural and Food Chemistry, 58, 6277-6284.

Guimarães, R., Barros, L., Carvalho, A. M., Ferreira, I. C. F. R. **(2010)**. Studies on chemical constituents and bioactivity of Rosa micrantha: Na alternative antioxidants source for food, pharmaceutical, or cosmetic applications. Journal of Agricultural and Food Chemistry, 58, 6277-6284.

Guimarães, R., Barros, L., Dueñas, M., Calhelha, R.C., Carvalho, A.M., Santos-Bulega, C., Queiroz, M.J.R.P., & Ferreira, I.C.F.R. **(2013)**. Nutrients, phytochemicals and bioactivity of wild Roman chamomile: a comparison between the herb and its preparations. Food Chemistry, 136, 718-725.

Guimarães, R., Barros, L., Dueñas, M., Carvalho, A. M., Queiroz, M. J. R. P., Santos-Buelga, C., Ferreira, I. C. F. R. **(2013)**. Characterization of phenolic compounds in wild fruits from Northeastern Portugal. Food Chemistry, 141, 3721-3730.

Gupta, V., Mittal, P., Bansal, P., Khokra, S.L., Kaushik, D. **(2010)**. Pharmacological potential of *Matricaria recutita* – A review. International Journal of Pharmaceutical Sciences and Drug research, 2, 12-16.

Hackett, A. M. **(1986)**. The metabolism of flavonoid compounds in mammals. Plant Flavonoids in biology and medicine: biochemical, pharmacological, and structure activity relationships. (Cody, V., Middleton, C., Jr & Harborne, J. B., eds). 177-194. Alan R. Liss, New York, NY.

Harborne, J. B., Grayer, R. J. **(1994)**. Flavonoids and insects. In: Harborne JB (ed) The flavonoids, advances in research since 1986. Chapman & Hall, London, pp 589–618.

Harborne, J. B., Williams, C.A. **(2000)**. Advances in flavonoid research since 1992. Phytochemistry, 55, 481-504.

Harbourne, N., Jacquier, J., O'Riordan, D. **(2009)**. Optimisation of the extraction and processing conditions of chamomile (*Matricaria chamomilla* L.) for incorporation into a beverage. Food Chemistry, 115, 15-19.

Hastam, E. **(1989)**. Plant polyphenols, Cambridge University. Press: Cambridge. U.K.

He, H., Chen, L., Zhai, M., Chen, J. Z. **(2009)**. Genistein down-regulates the constitutive activation of nuclear factor-kappaB in human multiple myeloma cells, leading to suppression of proliferation and induction of apoptosis. Phytotherapy Research, 23, 868-873.

Heim, K. E., Tagliaferro, A. R., Bobilya, D. J. **(2002)**. Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. Journal of Nutritional Biochemistry 13, 572–584

Heinrich, M. **(2003)**. Ethnobotany and Natural Products: The Search for New Molecules, New Treatments of Old Diseases or a Better Understanding of Indigenous Cultures? Current Topics in Medicinal Chemistry, 3, 29-34.

Herrman, K. J. **(1976)**. Flavonoids and flavones in food plants: A review. Journal of food technology, 11, 433-448.

Hirano, R., Sasamoto, W., Matsumoto, A., Itakura, J., Igarashi, O., Kondo, K. **(2001)**. Antioxidant ability of various flavonoids against DPPH radicals and LDL oxidation. Journal of Nutritional Science and Vitaminology, 47, 357-362.

Hollman, P. C. H. **(2004)**. Absorption, bioavailability and metabolism of flavonoids. Pharmaceutical biology, 42, 74-83.

Hollman, P. C. H., de Vries, J. H. M., Van Leeuwer, S. D., Memgelers, M. J. B., Katan, M. B. **(1995)**. Absorption of dietary quercetin glycosides and quercetin in healthy ileostomy volunteers. American Journal of Clinical Nutrition, 62, 1276-1278.

Hollman, P. C. H., Tijburg, L. B. M., Yang, C. S. **(1997)**. Bioavailability of flavonoids from tea. Critical Reviews in Food Science and Nutrition, 37, 719-738.

Hollman, P. C., Katan, M. B. **(1997)**. Absorption, metabolism and health effects of dietary flavonoids in man. Biomedicine & Pharmacotherapy, 51, 305-310.

Hraš, A.R., Halodin, M., Knez, Z., Bauman, D. **(2000)**. Comparison of antioxidative and synergistic effects of rosemary extract with α-tocopherol, ascorbyl palmitate and citric acid in sunflower oil. Food Chemistry, 71, 229-233.

Hvattum, E. **(2002)**. Determination of phenolic compounds in rose hip (*Rosa canina*) using liquid chromatography coupled to electrospray ionization tandem mass spectrometry and diode-array detection. Rapid Communications in Mass Spectrometry, 16, 655-662.

IUPAC. **(1976)**. Nomenclature of cyclitols. Biochemistry Journal, 153, 23-31.

Justino, G. C., Santos, M. R., Canario, S., Borges, C., Florencio, M. H., Mira, L. **(2004**). Plasma quercetin metabolites: structure-antioxidant activity relationships. Archives of Biochemistry Biophysics, 432, 109-121.

Kajjout, M., Rolando, C. **(2011)**. Regiospecific synthesis of quercetin O-β-d-glucosylated and O-β-d-glucuronidated isomers. Tetrahedron, 67, 4731-4741.

Kajjout, M., Zemmouri, R., Rolando, C. **(2011)**. An expeditious synthesis of quercetin-3-β-ɒ-glucuronide from rutin. Tetrahedron Letters, 52, 4738-4740.

Kanadaswami, C., Lee, L.-T, Lee, P.-P. H., Hwang, J.-J, Ke, F.-C., Huang, Y.-T., Lee, M.-T. **(2005**). The antitumor activities of flavonoids. *In vivo*, 19, 895-910.

Kayashima, T., Katayama, T. **(2002)**. Oxalic acid is available as a natural antioxidant in some systems. Biochimica et Biophysica Acta, 1573, 1-3.

Kharazmi, A. **(2008)**.Laboratory and preclinical studies on the anti-inflammatory ans anti-oxidant properties of rosehip powder – Identification and characterization of the active component GOPO®. Osteoarthritis and Cartilage, 16, 55-57.

King, R. A., Broadbent, J. L., Head, R. J. **(1996)**. Absorption of the soy isoflavone genestein in rats. Journal of Nutrition, 126, 176-182.

Koga, T., Meydani, M. **(2001)**. Effect of plasma metabolites of (+)-catechin and quercetin on monocyte adhesion to human aortic endothelial cells. The American Journal of Clinical Nutrition, 73, 941-948.

Kondo, K., Hirano, R., Matsumoto, A., Igarashi, O., Itakura, H. **(1996)**. Inhibition of LDL oxidation by cocoa. Lancet, 348, 1514-1518.

Kumar, S., Pandey, A. K. **(2013)**. Chemistry and biological activities of flavonoids: an overview. The scientific word journal. http://dx.doi.org/10.1155/2013/162750.

Lamarco, K. L., Glew, R. H. **(1986)**. Hydrolysis of a naturally occurring ß-glucoside by a broad specificity ß-glucoside from liver. Biochemistry, 237, 469-76.

Lin, L. Z., Harnly, J. M. **(2008)**. Identification of hydroxycinnamoylquinic acids of arnica flowers and burdock roots using a standardized LC-DAD-ESI/MS profiling method. Journal of Agriculture and Food Chemistry, 56, 10105-10114.

List, P.H., Horhammer, L. **(1971)**. Hager's handbuch der pharmazeutischen praxis. vols 2-6, 1969-1979. Springer-Verlag, Berlin.

Lotito, S. B., Zhang, W.-J., Yang, C. S., Crozier, A., Frei, B. **(2011)**. Metabolic conversion of dietary flavonoids alters their anti-inflammatory and antioxidant properties. Free Radical Biology and Medicine, 51, 454–463.

Lust, J. **(1980)**. The herb book. New York: Bantam.

Ma, C., Winsor, L., Daneshtalab, M. **(2007)**. Quantification of spiroether isomers an herniarin of different parts of *Matricaria matricarioides* and flowers of *Chamaemelum nobile*. Phytochemical Analysis, 18, 42-49.

Ma, T. E., Celestino, S., Julián, C. R. **(2004)**. Anthocyanins in cereals. Journal of Chromatography A, 1054, 129-141.

Mabry, T. J., Markham, K. R., Thomas, M. B. **(1970)**. The Systematic Identification of Flavonoids. New York: Springer-Verlag Publication.

Maldonado, P. D., Rivero-Cruz, I., Mata, R., Pedraza-Chaverri, J. **(2005)**. Antioxidant activity of A-type proanthocyanidins from *Geranium niveum* (Geraniaceae). Journal of Agricultural and Food Chemistry, 53, 1996-2001.

Manach, C., Morand, C., Crespy, V., Demigné, C., Texier, O., Régérat, F., Rémésy, C. **(1998)**. Quercetin is recovered in human plasma as conjugated derivatives which retain antioxidant properties. FEBS Letters, 426, 331-336.

Manach, C., Williamson, G., Morand, C., Scalbert, A., Rémésy, C. **(2005)**. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. The American Journal of Clinical Nutrition. 81(1 Suppl): 230S-242S.

Marles, M. A. S., Ray, H., Gruber, M. Y. **(2003)**. New perspectives on proanthocyanidin biochemistry and molecular regulation (review). Phytochemistry, 64, 367-383.

Maschi, O., Dal Cero, E., Galli, G. V., Caruso, D., Bosisio, E., Dell' Agli, M. **(2008)**. Inhibition of human cAMP-Phosphodiesterase as a mechanism of the spasmolytic effect of *Matricaria recutita* L. Journal of Agricultural and Food Chemistry, 56, 5015-5020.

Mazur, A., Bayle, D., Lab, C., Rock, E., Rayssiguier, Y. **(1999)**. Inhibitory effect of procyanidin-rich extracts on LDL oxidation *in vitro*. Artherosclerosis, 145, 421-422.

McKay, D. L., Blumberg, J. B. **(2006)**. A review of the bioactivity and potential health benefits of chamomile tea (*Matricaria recutita* L.). Phytotherapy Research, 20, 519-530.

Melegari, M., Albasini, A., Pecorari, P., Vampa, G., Rinaldi, M., Rossi, T., Bianchi, A. **(1988)**. Chemical characteristics and pharmacological properties of the essential oil of *Anthemis nobilis*. Fitoterapia, 59, 445–449

Mendes, L., Freitas, V., Baptista, P., Carvalho, M. **(2011)**. Comparative antihemolytiic and radical scavenging activities of strawberry tree (*Arbutus unedo* L.) leaf and fruit. Food and Chemical Toxicology, 49, 2285-2291

Middleton, E. **(1984)**. The flavonoids. Trends in pharmacological sciences, 5, 335-338.

Middleton, E. J. **(1998)**. Effect of plant flavonoids on immune and inflammatory cell function. Advances in Experimental Medicine and Biology, 439, 175-182.

Middleton, E. Jr., Chithan, K. **(1993)**. The impact of plant flavonoids on mammalian biology: Implications for immunity, inflammation and cancer. In: Harborne, J. B., editor. The flavonoids: advances in research since 1986, London, U.K: Chapman and Hall, 1993.

Middleton, E. Jr., Kanadaswami, C., Theoharides, T. C. **(2000)**. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease and cancer, Pharmacological Reviews, 52, 673-751.

Miliauskas, G., Venskutonis, P. R., van Beek, T. A. **(2004)**. Screening of radical scavenging activity of some medicinal and aromatic plant extracts. Food Chemistry, 85, 231-237

Miura, T., Chiba, M., Kasai, K., Nozaka, H., Nakamura, T., Shoji, T., Kanda, T., Ohtake, Y., Sato, T. **(2008)**. Apple procyanidins induce tumor cell apoptosis through mitochondrial pathway activation of caspase-3. Carcinogenesis, 29, 585-593.

Mladěnka, P., Zatloukalová, L., Filipský, T., Hrdina, R. **(2010)**. Cardiovascular effects of flavonoids are not caused only by direct antioxidant activity. Free Radical Biology & Medicine, 49, 963–975

Mojarrabi, B., Mackenzie, P. I. **(1998)**. Characterization of two UDP glucuronosyltransferases that are predominantly expressed in human colon. Biochemical and Biophysical Research Communications, 247, 704-709.

Moon, J. H., Tsushida, T., Nakahara, K., Terao, J. **(2001)**. Identification of quercetin 3-*O*-beta-D-glucuronide as an antioxidative metabolite in rat plasma after oral administration of quercetin. Free radical biology & medicine, 30, 1274-85.

Moon, Y. J., Wang, X., D., Morris, M. E. **(2006)**. Dietary flavonoids: effects on xenobiotic and carcinogen metabolism. Toxicology *in Vitro*, 20, 187-210.

Morales, P., Ferreira, I. C. F. R., Carvalho, A. M., Fernandéz-Ruiz, V., Camara, M., Morales, R., Tardio, J. **(2013)**. Wild edible fruits as a potential source of phytochemicals with capacity to inhibit lipid peroxidation. European Journal of Lipid Science and Technology, 115, 176-185.

Morishita, H., Ohnishi, M. **(2001)**. Absorption, metabolism and biological activities of chlorogenic acids and related compounds. Bioactive Natural Products, 25, Part F, 919-953.

Mukherjee, P., Shah, F., Desai, P., Avery, M. **(2011)**. Inhibitors of SARS-3CLpro: virtual screening, biological evaluation, and molecular dynamics simulation studies. Journal of chemical information and modeling, 51, 1376-92.

Mulinacci, N., Romani, A., Pinelli, P., Vincieri, F. F., Prucher, D. **(2000)**. Characterization of *Matricaria recutita* L. flower extracts by HPLC-MS and HPLC-DAD analysis. *Chromatographia*, *51*, 301-307.

Mullen, W., Edwards, C. A., Crozier, A. **(2006)**. Absorption, excretion and metabolite profiling of methyl-, glucuronyl-, glucosyl- and sulpho-conjugates of quercetin in human plasma and urine after ingestion of onions. British Journal of Nutrition, 96, 107-116.

Mullen, W., Rouanet, J. M., Auger, C., Teissèdre, P. L., Caldwell, S. T., Hartley, R. C., Lean, M. E., Edwards, C. A., Crozier, A. **(2008)**. Bioavailability of [2-(14)C] quercetin-4'-glucoside in rats. Journal of agricultural and food chemistry, 56, 12127-12137.

Nagata, M., Yamashita, I. **(1992)**. Simple method for simultaneous determination of chlorophyll and carotenoids in tomato fruit. Nippon Shokuhin Kogyo Gakkaish, 39, 925–928.

Narayana, K. R., Reddy, M. S., Chaluvadi, M. R., Krishna, D. R. **(2001)**. Bioflavonoids classification, pharmacological, biochemical effects and therapeutic potential. Indian journal of pharmacology, 33, 2-16.

Natsume, M., Osakabe, N., Oyama, M., Sasaki, M., Baba, S., Nakamura, Y., Osawa, T., Terao, J. **(2003)**. Structures of (-)-epicatechin glucuronide identified from plasma and urine after oral ingestion of (-)-epicatechin: differences between human and rat. Free Radical Biology and Medicine, 34, 840-849.

Nielsen, S. E., Breinholt, V., Cornett, C., Dragsted, L. O. **(2000)**. Biotransformation of the citrus flavone tangeretin in rays. Identification of metabolites with intact flavane nucleus. Food and Chemical Toxicology, 38, 739-746.

Nielsen, S. E., Breinholt, V., Justesen, U., Cornett, C., Dragsted, L. O. **(1998)**. *In vitro* biotransformation of flavonoids by rat liver microsomes. Xenobiotica, 28, 389–401.

Nijveld, R. J., van Nood, E., van Hoorn, D. E., Boelens, P. G., van Norren, K., van Leeuwen, P. A. (2001). Flavonoids: a review of probable mechanisms of action and potential applications. The American Journal of Clinical Nutrition, 74, 418-425.

Novais, H. M., Santos, I., Mendes, S., Pinto-Gomes, C. **(2004)**. Studies on pharmaceutical ethnobotany in Arrábida Natural Park. Journal of Ethnopharmacology, 93, 183–195.

Nováková, L., Vildová, A., Mateus, J. P., Gonçalves, T., Solich, P. **(2010)**. Development and application of UHPLC-MS/MS method for the determination of phenolic compounds in chamomile flowers and chamomile tea extracts. Talanta, 82, 1271-1280.

O'Boyle, N. M., Banck, M., James C. A., Morley, C., Vandermeersch, T., Hutchison, G. R. **(2011)**. Open Babel: An open chemical toolbox. Journal of Cheminformatics, 3, 33.

Olszewska, M., Wolbiś, M. **(2001)**. Flavonoids from flowers of *Prunus spinosa* L. Polish Pharmaceutical society, 58, 367-372.

Orhan, D. D., Hartevioğlu, A., Küpeli, E., Yesilalada, E. **(2007)**. *In vivo* anti-inflammatory and antinociceptive activity of the crude extract and fractions from *Rosa canina* L. fruits. Journal of Ethnopharmacology, 112, 394-400.

Osada, M., Imaoka, S., Funae, Y. **(2004)**. Apigenin suppresses the expression of VEGF, an important factor for angiogenesis, in endothelial cells via degradation of HIF-1α protein. FEBS Letters, 575, 59-63.

Pallauf, K., Rivas-Gonzalo, J. C., Castillo, M. D., Cano, M. P., Pascual-Teresa, S. **(2008)**. Characterization of the antioxidant composition of strawberry tree (*Arbutus unedo* L.) fruits. Journal of Food Composition and Analysis, 21, 273-281.

Pan, M.H., Lai, C.S., Wu, J.C., Ho, C.T. **(2011)**. Molecular mechanisms for chemoprevention of colorectal cancer by natural dietary compounds. Molecular Nutrition & Food Research, 55, 32-45.

Pandey, K. B., Rizvi, S. I. **(2009)**. Plant polyphenols as dietary antioxidants in human health and disease. Oxidative medicine and cellular longevity, 2, 270-178.

Parada, M., Carrió, E., Bonnet M. A., Vallès, J. **(2009)**. Ethnobotany of the Alt Empordà region (Catalonia, Iberian Peninsula). Plants used in human traditional medicine. Jornal of Ethnopharmacology, 124, 609-618.

Pawlowska, A. M., Leo, M., Braca, A. **(2006)**. Phenolics of *Arbutus unedo* L. (Ericaceae) fruits: Identification of anthocyanins and gallic acid derivatives. Journal of Agricultural and Food Chemistry, 54, 10234-10238.

Pereira, E., Barros, L., Martins, A., Ferreira, I. C. F. R. **(2012)**. Towards chemical and nutritional inventory of Portuguese wild edible mushrooms in different habitats. Food Chemistry, 130, 394-403.

Peterson, J., Dwyer, J. **(1998)**. Flavonoids: dietary occurrence and biochemical activity. Nutrition research, 12, 1995-2018.

Petronilho, S., Maraschin, M., Coimbra, M. A., Rocha, S. M. **(2012)**. *In vitro* and *in vivo* studies of natural products: A challenge for their valuation. The case study of chamomile (*Matricaria recutita* L.). Industrial Crops and Products, 40, 1-12.

Pieroni, A., Privietera, S. **(2014)**. Ethnobotany and its links to medical sciences and public health: quo vadis?. Zeitschrift für Phytotherapy, 35, 58-62.

Pietta, P. G., Mauri, P. L., Simonelti, P., Testolin, G., Fresenius, J. **(1995)**. HPLC and MEKC determination of major flavonoids in selected food pools. Journal of analytical chemistry, 352, 788-792.

Pietta, P. G. **(2000)**. Flavonoids as antioxidants. Journal of Natural Products, 63, 1035–1042.

Quave, C. L., Pardo-de-Santayana, M., Pieroni, A. **(2012)**. Medical Ethnobotany in Europe: From Field Ethnography to a More Culturally Sensitive Evidence-Based CAM? Evidence-Based Complementary and Alternative Medicine. ID 156846, 17 pages. http://dx.doi.org/10.1155/2012/156846.

Ramos, S. **(2007)**. Effects of dietary flavonoids on apoptotic pathaways related to cancer chemoprevention. Journal Nutritional Biochemistry, 18, 427-442.

Rao, K. V., Owoyale, J. A. **(1976)**. Partial methylation of quercetin: Direct synthesis of tamarixetin, ombuin and ayanin. Journal of heterocyclic chemistry, 13, 1293-1295.

Rein, E., Kharazmi, A., Winther, K. **(2004)**. A herbal remedy, Hyben Vital (stand. powder of a subspecies of *Rosa canina* fruits), reduces pain and improves general wellbeing in patients with osteoarthritis—a double-blind, placebo-controlled, randomised trial. Phytomedicine, 11, 383-391.

Rice-Evans, C. **(2001)**. Flavonoid antioxidants. Current Medicinal Chemistry, 8, 797-808.

Rossi, T., Melegari, M., Bianchi, A., Albasini, A., Vampa, G. **(1988)**. Sedative, anti-inflammatory and anti-diuretic effects induced in rats by essential oils of varieties of *Anthemis nobilis*: A comparative study..Pharmaceutical Research, 20, 71–74.

Rothmaler, W. **(2007)**. Exkursionsflora von Deutschland. Gefaesspflanzen: Atlasband, Band 3. Elsevier GmbH Munchen.

Salgueiro, J. **(2004)**. *Ervas, usos e saberes*. Lisboa: MARCA, Associação de Desenvolvimento Local.

Salminen, J. P., Karonen, M., Lempa, K., Liimatainen, J., Sinkkonen, J., Lukkarinen, M., Pihlaja, K. **(2005)**. Characterisation of proantocyanidin aglycones and glycosides from rose hips by high-performance liquid chromatography-mass spectrometry, and their rapid quantification together with Vitamin C. Journal of Chromatography A, 1077, 170-180.

Sanner, M. F. **(2005)**. A component-based software environment for visualizing large macromolecular assemblies. Structure, 13, 447-462.

Santos-Buelga, G., Garcia-Viguera, C., Tomas-Barberan, A. **(2003)**. On-line identification of flavonoids by HPLC coupled to diode array detection. In Methods in Polyphenol Analysis, (pp. 92–128). Cambridge: Royal Society of Canada.

Scalbert, A. **(1991)**. Antimicrobial properties of tannins (review). Phytochemistry, 30, 3875-3883.

Scalbert, A., Morand, C., Manach, C., Rémésy, C. **(2002)**. Absorption and metabolism of polyphenols in the gut and impact on health. Biomedicine and Pharmacotherapy, 56, 276-282.

Schempp, H., Weiser, D., Kelber, O., Elstner, E. F. **(2006)**. Radical scavenging and anti-inflammatory properties of STW 5 (Iberogasts) and its components. Phytomedicine, 13, 36-44.

Schroeter, H., Spencer, J. P. E., Rice-Evans, C., Williams, R. J. **(2001)**. Flavonoids protect neurons from oxidized low-density-lipoprotein-induced apoptosis involving c-Jun N-terminal kinase (JNK), c-Jun and caspase-3. Biochemical Journal, 358, 547-557.

Setchell, K. D. R., Faughnan, M. S., Avades, T., Zimmer-Nechemias, L., Brown, N. M., Wolfe, B. E., Brashear, W. T., Desai, P., Oldfield, M. F., Botting, N. P., Cassidy, A. **(2003)**. Comparing the pharmacokinetics of daidzein and genistein with the use of ^{13}C-labeled tracers in premenopausal women. The American Journal of Clinical Nutrition, 77, 411-419.

Shahat, A. A., Cos, P., De Bruyne, T., Apers, S., Hammouda, F. M., Ismail, S. I., Azzam, S., Claeys, M., Goovaerts, E., Pieters, L., Vanden Berghe, D., Vlietinck, A. J. **(2002)**. Antiviral and antioxidant activity of flavonoids and proanthocyanidins from *Crataegus sinaica*. Planta Medica, 68, 539-541.

Shi, Z**.** H**.,** Li**,** N**.** G**.,** Tang**,** Y**.** P**.,** Li, W., Yin, L., Yang, J. P., Tang, H., Duan, J. A. (**2012).** Metabolism-based synthesis, biologic evaluation and SARs analysis of *O*-methylated analogs of quercetin as thrombin inhibitors**.** European Journal of Medicinal Chemistry, 54, 210-222**.**

Shih, H., Pickwell, G.V., Quattrochi, L.C. **(2000)**. Differential effects of flavonoid compounds on tumor promoter-induced activation of the human CYP1A2 enhancer. Archives of Biochemistry and Biophysics, 373, 287–294

Silva, D. I., Rodrigues, A. S., Gaspar, J., Laires, A., Rueff, J. **(1997)**. Metabolism of galangin by rat cytochromes P450: relevance to the genotoxicity of galangin. Mutation Research, 393, 247–257.

Silva, D. I., Rodrigues, A. S., Gaspar, J., Maia, R., Laires, A., Rueff, J. **(1997)**. Involvement of rat cytochrome 1A1 in the biotransformation of kaempferol to quercetin: relevance to the genotoxicity of kaempferol. Mutagenesis, 12, 383-390.

Škerget, M., Kotnik, P., Hadolin, M., Hraš, A. R., Simonič, M., Knez, Ž. **(2005)**. Phenols, proanthocyanidins, flavones and flavonols in some plant materials and their antioxidant activities. Food chemistry, 89, 191-198.

Soares, P., Costa, R., Froufe, H. J. C., Calhelha, R. C., Peixoto, D., Ferreira, I. C. F. R., Abreu, R. M. V., Soares, R., Queiroz M. J. R. P. **(2013)**. 1-Aryl-3-[4-(thieno[3,2-*d*]pyrimidin-4-yloxy)phenyl]ureas as VEGFR-2 tyrosine kinase inhibitors: synthesis, biological evaluation and molecular modelling studies". BioMed Research International vol. 2013, Article ID 154856, 9 pages.

Spencer, J. P. E., Vouzour, D., Rendeiro, C. **(2009)**. Flavonoids and cognition: the molecular mechanisms underlying their behavioral effects. Archives of biochemistry and biophysics, 492, 1-9.

Srivastava, J. K., Gupta, S. **(2007)**. Antiproliferative and apoptotic effects of chamomile extract in various human cancer cells. Journal of Agricultural and Food Chemistry, 55, 9470-9478.

Srivastava, J. K., Gupta, S. **(2009)**. Extraction, characterization, stability and biological activity of flavonoids isolated from chamomile flowers. Molecular Cell Pharmacology, 1, 138-152.

Srivastava, J.K., Shankar, E., Gupta, S. **(2010)**. Chamomile: A herbal medicine of the past with a bright future (Review). Molecular Medicine Reports, 3, 895-901.

Stagos, D., Amoutzias, G. D., Matakos, A., Spyrou, A., Tsatsakis, A. M., Kouretas, D. **(2012)**. Chemoprevention of liver cancer by plant polyphenols. Food and Chemical Toxicology, 50, 2155-2170.

Staub, P. O., Geck, M. S., Weckerle, C. S., Casu, L., Leonti, M. **(2015)**. Classifying diseases and remedies in ethnomedicine and ethnopharmacology. Journal of Ethnopharmacology, 174, 514-519.

Staub, P. O., Geck, M. S., Weckerle, C. S., Casu, L., Leonti, M. **(2015)**. Classifying diseases and remedies in ethnomedicine and ethnopharmacology. Journal of Ethnopharmacology, 174, 514-519.

Stobiecki, M., Kachlicki, P. **(2006)**. Isolation and identification of flavonoids. In The Science of flavonoids, Edited by Erich Growteworld, The Ohio state University, Columbus, Ohio, USA, Springer. ISBN-10:0-387-28821-X.

Strassburg, C. P., Nguyen, N., Manns, M. P., Tukey, R. H. **(1998)**. Polymorphic expression of the UDP-glucuronosyltransferase UGT1A gene locus in human gastric epithelium. Molecular Pharmacology, 54, 647-654.

Strassburg, C. P., Nguyen, N., Manns, M. P., Tukey, R. H. **(1999)**. UDP glucuronosyltransferase activity in human liver and colon. Gastroenterology, 116, 149-160.

Tavares, L., Fortalezas, S., Carrilho, C., McDougall, G. J., Stewart, D., Ferreira, R. B., Santos, C. N. **(2010)**. Antioxidant and antiproliferative properties of strawberry tree tissues. Journal of Berry Research, 1, 3-12.

Terra, X., Valls, J., Vitrac, X., Merrillon, J. M., Arola, L., Ardevol, A., Blade, C., Fernandez-Larrea, J., Pujadas, G., Salvado, J., Blay, M. **(2007)**. Grape-seed procyanidins act as anti-inflammatory agents in endotoxin-stimulated RAW 264.7 macrophages by inhibiting NFkB signalling pathway. Journal of Agricultural and Food Chemistry, 55, 4357-4365.

Tolouee, M., Alinezhad, S., Saberi, R., Eslamifar, A., Zad, S. J., Jaimand, K., Taeb, J., Rezaee, M. B., Kawachi, M., Shams-Ghahfarokhi, M., Razzaghi-Abyaneh, M. **(2010)**. Effect of *Matricaria chamomilla* L. flower essential oil on the growth and ultrastructure of *Aspergillus niger* van Tieghem. International Journal of Food Microbiology, 139, 127–133.

Trott, O., Olson, A. J. **(2010)**. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization and multithreading. Journal of Computational Chemistry 31, 455-461.

Tschan, G. M., Konig, G. M., Wright A. D., Stichert, O. **(1996)**. Chamaemeloside, a new flavonoid glycoside from *Chamaemelum nobile*. Phytochemistry, 41, 643-646.

Tumbas, V. T., Čanadanović-Brunet, J. M., Četojević-Simin, D. D., Ćetković, G. S., Dilas, S. M., Gille, L. **(2012)**. Effect of rosehip (*Rosa canina* L.) phytochemicals on stable free radicals and human cancer cells. Journal of the Science of Food and Agriculture, 92, 1273-1281.

Uggla, M., Gustavsson, K.-E., Olsson, M. E., Nybom, H. **(2005)**. Changes in colour and sugar contentin rose hips (*Rosa dumalis* L., and *R. rubiginosa* L.) during ripening. Journal of Horticultural Science and Biotechnology; 80, 204-208.

Upadhyay, S., Dixit, M. **(2015)**. Role of Polyphenols and Other Phytochemicals on Molecular Signaling. Oxidative Medicine and Cellular Longevity, http://dx.doi.org/10.1155/2015/504253.

Van der Woude, H., Boersma, M. G., Vervoort, J., Rietjens, I. M. **(2004)**. Identification of 14 quercetin phase II mono- and mixed conjugates and their formation by rat and

human phase II *in vitro* model systems. Chemical Research in Toxicology, 17, 1520-1530.

Walle, T. **(2004)**. Absorption and metabolism of flavonoids. Free Radical Biology & Medicine, 36, 829-837.

Wang, H., Cao, G., Prior, R. L. **(1997)**. Oxygen radical absorbing capacity of anthocyanins. Journal of Agricultural and Food Chemistry, 45, 304–309.

Wasilewski, B., Czelej, J. **(1994)**. Leksykon leków homeopatycznych. Wydawnictwo SPLIT TRADING, Warszawa.

Weber, J. M., Ruzindana-Umunyana, A., Imbeault, L., Sircar, S. **(2003).** Inhibition of adenovirus infection and adenain by green tea catechins. Antiviral research, 58, 167-173.

Weiss, R. F. **(1988)**. Herbal Medicine. Arcanum AB (ed). Beaconsfield Publishers, Beaconsfield, 22-28.

Widlansky, M. E., Duffy, S. J., Hamburg, N. M., Gokce, N., Warden, B. A., Wiseman, S., Keaney, J. F, Jr., Frei, B., Joseph, A., Vita, J. A. **(2005)**. Effects of black tea consumption on plasma catechins, markers oxidative stress and inflammation in patients with coronary artery disease. Free Radical Biology and Medicine, 38, 499-506.

Williams, R. J., Spencer, J. P. E., Rice-Evans, C. **(2004)**. Flavonoids: antioxidants or signalling molecules? Free Radical Biology and Medicine, 36, 838–849.

Winkel-Shirley, B. **(1996)**. Flavonoid biosynthesis: 'new' functions for an 'old' pathway. Trends in plants Science, 1, 377-382.

Winkel-Shirley, B. **(2001)**. Flavonoid biosynthesis: a colorful model for genetics, biochemistry cell biology, and biotechnology. Plant physiology, 126, 485-493.

Wissemann, V., Gallenmüller, F., Ritz, C., Steinbrecher, T., Speck, T. **(2006)**. Inheritance of growth form and mechanical characters in reciprocal polyploid hybridsof Rosa section - implications for the ecological niche differentia-tion and radiation process of hybrid offspring. Trees (Berl West), 20, 340–347.

Yang, C. S., Chen, L. S., Lee, M. J., Balentine, D., Kuo, M. C., Schantz, S. P. **(1998)**. Blood and urine levels of tea catechins after ingestion of different amounts of green tea by human volunteers. Cancer Epidemiology, Biomarkers & Prevention, 7, 351-354.

Yao, L. H., Jiang, Y. M., Shi, J., Tomás-Barberán, F. A., Datta, N., Singanusong, R., Chen, S. S. **(2004)**. Flavonoids in food and their health benefits. Plant foods Human Nutrition, 59, 113-122.

Yildiz, O., Alpaslan, M. **(2012)**. Properties of rose hip marmalades. Food Technology and Biotechnology, 50, 98–106.

Yoshizumi, M., Tsuchiya, K., Kirima, K., Kyaw, M., Suzaki, Y., Tamaki, T. **(2001)**. Quercetin inhibits Shc- and Phosphatidylinositol 3-kinase-mediated c-Jun N-terminal kinase activation by angiotensin II in cultured rat aortic smooth muscle cells. Molecular Pharmacology, 60, 656–665.

Zhang, Y., Hendrich, S., Murphy, P. A. **(2003)**. Glucuronides are the main isoflavone metabolites in women. Journal of Nutrition, 133, 399-404.

Zhao, R., Xiang, N., Domann, F.E., Zhong, W. **(2009)**. Effects of selenite and genistein on G2/M cell cycle arrest and apoptosis in human prostate cancer cells. Nutrition and Cancer, 61, 397-407.

Ziyyat, A., Boussairi, E. **(1998)**. Cardiovascular effects of Arbutus unedo L. in spontaneously hypertensive rats. Phytotherapy Research, 12, 110–113.

Ziyyat, A., Legssyer, A., Mekhfi, H., Dassouli, A., Serhrouchni, M., Benjelloun, W. **(1997)**. Phytotherapy of hypertension and diabetes in oriental Morocco. Journal of Ethnopharmacology, 58, 45–54.

Ziyyat, A., Mekhfi, H., Bnouham, M., Tahri, A., Legssyer, A., Hoerter, J., Fischmeister, R. **(2002)**. *Arbutus unedo* induces endothelium-dependent relaxation of the isolated rat aorta. Phytotherapy Research, 16, 572–575.

Chapter 7. Published work

7.1 “Nutrients, phytochemicals and bioactivity of wild Roman chamomile: a comparison between the herb and its preparations”

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Nutrients, phytochemicals and bioactivity of wild Roman chamomile: A comparison between the herb and its preparations

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ABSTRACT

Roman chamomile, *Chamaemelum nobile* L. (Asteraceae), has been used for medicinal applications, mainly through oral dosage forms (decoctions and infusions). Herein, the nutritional characterisation of *C. nobile* was performed, and herbal material and its decoction and infusion were submitted to an analysis of phytochemicals and bioactivity evaluation. The antioxidant activity was determined by free radicals scavenging activity, reducing power and inhibition of lipid peroxidation, the antitumour potential was tested in human tumour cell lines (breast, lung, colon, cervical and hepatocellular carcinomas), and the hepatotoxicity was evaluated using a porcine liver primary cell culture. *C. nobile* proved to be an equilibrated valuable herb rich in carbohydrates and proteins, and poor in fat, providing tocopherols, carotenoids and essential fatty acids (C18:2n6 and C18:3n3). Moreover, the herb and its infusion are a source of phenolic compounds (flavonoids such as flavonols and flavones, phenolic acids and derivatives) and organic acids (oxalic, quinic, malic, citric and fumaric acids) that showed antioxidant and antitumour activities, without hepatotoxicity. The most abundant compounds in the plant extract and infusion were 5-*O*-caffeoylquinic acid and an apigenin derivative. These, as well as other bioactive compounds, are affected in *C. nobile* decoction, leading to a lower antioxidant potential and absence of antitumour potential. The plant bioactivity could be explored in the medicine, food, and cosmetic industries.

1. Introduction

Roman chamomile, *Chamaemelum nobile* L. (Asteraceae), is a perennial herb found in wild and cultivated habitats in western Europe, North America and northern Africa. Traditionally chamomile is considered to be an antiseptic, antibiotic, disinfectant, bactericidal, fungicidal and vermifuge. It has been used for centuries as anti-inflammatory, antioxidant, mild astringent, mild sedative, antispasmodic, antibacterial and healing medicine (Ma, Winsor, & Daneshtalab, 2007). Oral dosage forms (decoctions and infusions) are used for the symptomatic treatment of gastrointestinal disorders and of the painful component of functional digestive symptoms. External applications of extracts and lotions are recommended as repellent, emollient, in the treatment of skin disorders and for eye irritation or discomfort of various etiologies. Furthermore, it is used as an analgesic in diseases of the oral cavity, oropharynx or both and as a mouthwash for oral hygiene (Srivastava, Shankar, & Gupta, 2010). Different classes of bioactive constituents are present in chamomile, including phenolic compounds (Carnat, Carnat, Fraisse, Ricoux, & Lamaison, 2004; Tschan, Konig, Wright, & Stichert, 1996).

Phenolic compounds have the capacities to quench lipid peroxidation, prevent DNA oxidative damage, and scavenge reactive oxygen species (ROS), such as superoxide, hydrogen peroxide, and hydroxyl radicals (Cao & Cao, 1999). Flavonoids are the most abundant antioxidants found in common diets (Mladěnka, Zatloukalová, Filipský, & Hrdina, 2010). The benefits of flavonoids on human health are very often ascribed to their potential ability to act diminishing free radical steady state concentration in biological systems providing antioxidant protection (Galleano, Verstraenten, Oteiza, & Fraga, 2010). Such ability could be possible considering that polyphenols have chemical structures supporting the scavenging of free radicals and the chelation of redox-active metals. In parallel, it has been reported that certain flavonoids can provide benefits in pathological situations associated with high free radical production, (e.g. hypertension, cardiovascular and cancer diseases) (Galleano et al., 2010; Grassi et al., 2009; Schroeter et al., 2006). In fact, phenolic compounds, mainly flavonoids, proved to have the capacity of regulating proliferation and cell death pathways leading to cancer (López-Lázaro, 2002), through different mechanisms including cell growth and kinase activity inhibition, apoptosis induction, suppression of the secretion of

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matrix metalloproteinases and of tumour invasive behaviour, as well as angiogenesis impairment (Kandaswami et al., 2005).

In the present work, the nutritional characterisation (macronutrients, free sugars, fatty acids, tocopherols and carotenoids) of *C. nobile* was performed, and the herb and its decoction and infusion were submitted to a detailed analysis of phytochemicals (phenolic compounds and organic acids) and bioactivity evaluation. The antioxidant activity was determined by free radicals scavenging activity, reducing power and inhibition of lipid peroxidation, the antitumour potential was tested in human tumour cell lines (breast, lung, colon, cervical and hepatocellular carcinomas), and the hepatotoxicity was evaluated using a porcine liver primary cell culture.

2. Materials and methods

2.1. Sample

C. nobile was gathered during the flowering season (June–July, 2010) from wild populations located in grasslands in Bragança (Trás-os-Montes, Northeastern Portugal), considering the local medicinal uses as well as healers and selected consumers criteria, which are related to particular gathering sites, and requirements for safe herbal dosage forms, such as infusion and decoction.

Samples consisted of pieces of about 8 cm, corresponding to terminal soft leafy stems and inflorescences with flowers fully open and functional, which were randomly selected in a meadow of about a hectare. The plant material was put together in a single sample for analysis. Voucher specimens were deposited in the Herbarium of the Escola Superior Agrária de Bragança (BRESA). The sample was lyophilised (FreeZone 4.5, Labconco, Kansas, USA), reduced to a fine dried powder (20 mesh) and mixed to obtain homogenate sample.

2.2. Standards and reagents

Acetonitrile 99.9%, *n*-hexane 95% and ethyl acetate 99.8% were of HPLC grade from Fisher Scientific (Lisbon, Portugal). The fatty acids methyl ester (FAME) reference standard mixture 37 (standard 47885-U) was purchased from Sigma (St. Louis, MO, USA), as also other individual fatty acid isomers, sugars (D-(−)-Fructose, D-(+)-Glucose, D-(+)-Sucrose, D-(+)-Trehalose), tocopherols (α-, β-, γ-, and δ-isoforms) and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) standards. Racemic tocol, 50 mg/ml, was purchased from Matreya (PA, USA). The phenolic compound standards (apigenin-6-*C*-glucoside; apigenin-7-*O*-glucoside; caffeic acid; chlorogenic acid; *p*-coumaric acid; isorhamnetin-3-*O*-glucoside; kaempferol-3-*O*-rutinoside; luteolin-6-*C*-glucoside; luteolin-7-*O*-glucoside; myricetin-3-*O*-glucoside; protocatechuic acid; quercetin 3-*O*-glucoside and quercetin-3-*O*-rutinoside) were from Extrasynthese (Genay, France). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Foetal bovine serum (FBS), L-glutamine, hank's balanced salt solution (HBSS), trypsin-EDTA (ethylenediaminetetraacetic acid), penicillin/streptomycin solution (100 U/ml and 100 mg/ml, respectively), RPMI-1640 and DMEM media were from Hyclone (Logan, USA). Acetic acid, ellipticine, sulforhodamine B (SRB), trypan blue, trichloroacetic acid (TCA) and Tris were from Sigma Chemical Co. (St. Louis, USA). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

2.3. Characterisation of plant nutrients

2.3.1. Crude composition

The sample was analysed for chemical composition (moisture, proteins, fat, carbohydrates and ash) using the AOAC procedures (AOAC, 1995). The crude protein content (N × 6.25) was estimated by the macro-Kjeldahl method; the crude fat was determined by extracting a known weight of powdered sample with petroleum ether, using a Soxhlet apparatus; the ash content was determined by incineration at 600 ± 15 °C. Total carbohydrates were calculated by difference. Energy was calculated according to the following equation: Energy (kcal) = 4 × (g protein + g carbohydrate) + 9 × (g fat).

2.3.2. Sugars composition

Free sugars were determined by a High Performance Liquid Chromatography (HPLC) system consisting of an integrated system with a pump (Knauer, Smartline system 1000), degasser system (Smartline manager 5000) and auto-sampler (AS-2057 Jasco), coupled to a refraction index detector (RI detector Knauer Smartline 2300) as previously described by the authors (Pereira, Barros, Martins, & Ferreira, 2012). Sugars identification was made by comparing the relative retention times of sample peaks with standards. Data was analysed using Clarity 2.4 Software (DataApex). Quantification was based on the RI signal response of each standard, using the internal standard (IS, raffinose) method and by using calibration curves obtained from commercial standards of each compound. The results were expressed in g per 100 g of dry weight.

2.3.3. Fatty acids composition

Fatty acids were determined after a transesterification procedure as described previously by the authors (Pereira et al., 2012), using a gas chromatographer (DANI 1000) equipped with a split/splitless injector and a flame ionisation detector (GC–FID). Fatty acid identification was made by comparing the relative retention times of FAME peaks from samples with standards. The results were recorded and processed using CSW 1.7 software (DataApex 1.7). The results were expressed in relative percentage of each fatty acid.

2.3.4. Tocopherols composition

Tocopherols were determined following a procedure previously optimised and described by the authors (Pereira et al., 2012). Analysis was performed by HPLC (equipment described above), and a fluorescence detector (FP-2020; Jasco) programmed for excitation at 290 nm and emission at 330 nm. The compounds were identified by chromatographic comparisons with authentic standards. Quantification was based on the fluorescence signal response of each standard, using the IS (tocol) method and by using calibration curves obtained from commercial standards of each compound. The results were expressed in mg per 100 g of dry weight.

2.3.5. Carotenoids

β-Carotene and lycopen were determined following a procedure previously described by Nagata and Yamashita (1992). A fine dried powder (500 mg) was vigorously shaken with 10 ml of acetone–hexane mixture (4:6) for 1 min and filtered through Whatman No. 4 filter paper. The absorbance of the filtrate was measured at 453, 505, 645 and 663 nm. Content of β-carotene and lycopene were calculated according to the following equations:

$$\beta\text{-carotene (mg/100 ml)} = 0.216 \times A_{663} - 1.220 \times A_{645} - 0.304 \times A_{505} + 0.452 \times A_{453}; \quad (1)$$

$$\text{Lycopene (mg/100 ml)} = -0.0458 \times A_{663} + 0.204 \times A_{645} - 0.304 \times A_{505} + 0.452 \times A_{453}; \quad (2)$$

and further expressed in mg per 100 g of dry weight (dw).

2.4. Analysis of phytochemicals in the herb and in its decoction and infusion

2.4.1. Plant extraction

For bioactivity assays, a methanolic extract was prepared from the lyophilised plant material. The sample (1 g) was extracted by stirring with 25 ml of methanol (25 °C at 150 rpm) for 1 h and subsequently filtered through Whatman No. 4 paper. The residue was then extracted with 25 ml of methanol (25 °C at 150 rpm) for 1 h. The combined methanolic extracts were evaporated at 40 °C (rotary evaporator Büchi R-210) to dryness.

2.4.2. Decoction preparation

The sample (1 g) was added to 200 ml of distilled water, heated (heating plate, VELP scientific) and boiled for 5 min. The mixture was left to stand at room temperature for 5 min more, and then filtered under reduced pressure. The obtained decoction was frozen and lyophilised.

2.4.3. Infusion preparation

The sample (1 g) was added to 200 ml of boiling distilled water and left to stand at room temperature for 5 min, and then filtered under reduced pressure. The obtained infusion was frozen and lyophilised.

2.4.4. Organic acids composition

Organic acids were determined following a procedure previously optimised and described by the authors (Barros, Pereira, & Ferreira, 2012). Analysis was performed by ultra fast liquid chromatography (UFLC) coupled to photodiode array detector (PDA), using a Shimadzu 20A series UFLC (Shimadzu Corporation). Detection was carried out in a PDA, using 215 nm and 245 as preferred wavelengths. The organic acids were quantified by comparison of the area of their peaks recorded at 215 nm with calibration curves obtained from commercial standards of each compound. The results were expressed in g per 100 g of lyophilised methanolic extract/decoction/infusion.

2.4.5. Phenolic compounds composition

Phenolic compounds were determined by HPLC (Hewlett-Packard 1100, Agilent Technologies, Santa Clara, USA) as previously described by the authors (Barros et al., 2012). Double online detection was carried out in the diode array detector (DAD) using 280 and 370 nm as preferred wavelengths and in a mass spectrometer (API 3200 Qtrap, Applied Biosystems, Darmstadt, Germany) connected to the HPLC system via the DAD cell outlet. The phenolic compounds were characterised according to their UV and mass spectra and retention times, and comparison with authentic standards when available. For quantitative analysis, calibration curves were prepared from different standard compounds. The results were expressed in g per 100 g of lyophilised methanolic extract/decoction/infusion.

2.5. Evaluation of bioactivity

2.5.1. General

The lyophilised methanolic extract, decoction and infusion were redissolved in (i) water and methanol, respectively (final concentration 2.5 mg/ml) for antioxidant activity evaluation, or (ii) water for antitumour activity evaluation (final concentration 8 mg/ml). The final solutions were further diluted to different concentrations to be submitted to distinct bioactivity evaluation *in vitro* assays. The results were expressed in (i) EC_{50} values (sample concentration providing 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay) for antioxidant activity, or (ii) GI_{50} values (sample concentration that inhibited 50% of the net cell growth) for antitumour activity. Trolox and ellipticine were used as standards in antioxidant and antitumour activity evaluation assays, respectively.

2.5.2. Antioxidant activity

DPPH radical-scavenging activity was evaluated by using an ELX800 microplate reader (Bio-Tek Instruments, Inc.; Winooski, USA), and calculated as a percentage of DPPH discolouration using the formula: $[(A_{DPPH} - A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution containing the sample at 515 nm, and A_{DPPH} is the absorbance of the DPPH solution. Reducing power was evaluated by the capacity to convert Fe^{3+} into Fe^{2+}, measuring the absorbance at 690 nm in the microplate reader mentioned above. Inhibition of β-carotene bleaching was evaluated though the β-carotene/linoleate assay; the neutralisation of linoleate free radicals avoids β-carotene bleaching, which is measured by the formula: (β-carotene absorbance after 2 h of assay/initial absorbance) × 100. Lipid peroxidation inhibition in porcine (*Sus scrofa*) brain homogenates was evaluated by the decreasing in thiobarbituric acid reactive substances (TBARS); the colour intensity of the malondialdehyde–thiobarbituric acid (MDA–TBA) was measured by its absorbance at 532 nm; the inhibition ratio (%) was calculated using the following formula: $[(A - B)/A] \times 100\%$, where A and B were the absorbance of the control and the sample solution, respectively (Pereira et al., 2012).

2.5.3. Antitumour activity

Five human tumour cell lines were used: MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer), HCT-15 (colon carcinoma), HeLa (cervical carcinoma) and HepG2 (hepatocellular carcinoma). Cells were routinely maintained as adherent cell cultures in RPMI-1640 medium containing 10% heat-inactivated FBS (MCF-7, NCI-H460 and HCT-15) and 2 mM glutamine or in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin (HeLa and HepG2 cells), at 37 °C, in a humidified air incubator containing 5% CO_2. Each cell line was plated at an appropriate density (7.5×10^3 cells/well for MCF-7, NCI-H460 and HCT-15 or 1.0×10^4 cells/well for HeLa and HepG2) in 96-well plates and allowed to attach for 24 h. Cells were then treated for 48 h with various extract concentrations. Following this incubation period, the adherent cells were fixed by adding cold 10% trichloroacetic acid (TCA, 100 μl) and incubated for 60 min at 4 °C. Plates were then washed with deionised water and dried; sulforhodamine B solution (0.1% in 1% acetic acid, 100 μl) was then added to each plate well and incubated for 30 min at room temperature. Unbound SRB was removed by washing with 1% acetic acid. Plates were air dried, the bound SRB was solubilised with 10 mM Tris (200 μl) and the absorbance was measured at 540 nm in the microplate reader mentioned above.

2.5.4. Hepatotoxicity

A cell culture was prepared from a freshly harvested porcine liver obtained from a local slaughter house, and it was designed as PLP2. Briefly, the liver tissues were rinsed in hank's balanced salt solution containing 100 U/ml penicillin, 100 μg/ml streptomycin and divided into 1×1 mm^3 explants. Some of these explants were placed in 25 cm^2 tissue flasks in DMEM medium supplemented with 10% foetal bovine serum, 2 mM nonessential amino acids and 100 U/ml penicillin, 100 mg/ml streptomycin and incubated at 37 °C with a humidified atmosphere containing 5% CO_2. The medium was changed every 2 days. Cultivation of the cells was continued with direct monitoring every two to three days using a phase contrast microscope. Before confluence was reached, cells were subcultured and plated in 96-well plates at a density of 1.0×10^4 cells/well, and cultivated in DMEM medium with 10%

FBS, 100 U/ml penicillin and 100 μg/ml streptomycin (Abreu et al., 2011).

2.6. Statistical analysis

All the assays were carried out in triplicate in three different samples, and the results are expressed as mean values ± standard deviation (SD). The statistical differences represented by letters were obtained through one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference post hoc test with $\alpha = 0.05$. These treatments were carried out using SPSS v. 18.0 program.

3. Results and discussion

3.1. Characterisation of plant nutrients

The results of the nutritional characterisation of *C. nobile* are shown in Table 1. Carbohydrates were the most abundant macronutrients, followed by proteins. Ash and fat contents were low, and the energetic contribution was 389.88 kcal/100 g dw. The main sugar found in this plant material was fructose, followed by glucose and sucrose. Trehalose was found in lower amounts. Polyunsaturated fatty acids (PUFA) predominated over saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA). The FA determined in higher percentages, were linoleic acid (C18:2n6), oleic acid (C18:1n9), α-linolenic acid (C18:3n3) and palmitic acid (C16:0). Regarding tocopherols, only α- and γ-tocopherols were found in the studied plant material. β-Carotene and lycopene were also quantified in the studied sample.

As far as we know this is the first report on nutritional characterisation of *C. nobile*, that proved to be an equilibrated valuable herb rich in carbohydrates and proteins, and poor in fat and calories. Moreover, it provides tocopherols, carotenoids, essential fatty acids (C18:2n6 and C18:3n3), and ratios PUFA/SFA and n-6/n-3 fatty acids higher than 0.45 and lower than 4.0, respectively (Guil, Torija, Giménez, & Rodriguez, 1996), which are considered good nutritional ratios.

Table 1
Nutritional characterisation of *Chamaemelum nobile* (mean ± SD).

Crude composition (g/100 g dw)		Free sugars (g/100 g dw)	
Moisture (g/100 g fw)	67.09 ± 1.02	Fructose	3.37 ± 0.24
Ash	6.43 ± 0.05	Glucose	1.57 ± 0.13
Proteins	26.63 ± 1.92	Sucrose	1.08 ± 0.08
Fat	3.12 ± 0.33	Trehalose	0.60 ± 0.02
Carbohydrates	63.83 ± 1.66	Total sugars	6.62 ± 0.31
Energy	389.88 ± 1.32		
Fatty acids (relative %)		*Tocopherols (mg/100 g dw)*	
C16:0	17.89 ± 0.16	α-Tocopherol	1.64 ± 0.02
C18:0	3.36 ± 0.03	β-Tocopherol	nd
C18:1n9	23.22 ± 0.22	γ-Tocopherol	0.19 ± 0.01
C18:2n6	28.89 ± 0.33	δ-Tocopherol	nd
C18:3n3	18.22 ± 0.11	Total tocopherols	1.83 ± 0.01
SFA	27.67 ± 0.19	*Pigments (mg/100 g)*	
MUFA	24.78 ± 0.27	β-Carotene	0.95 ± 0.02
PUFA	47.56 ± 0.46	Lycopene	0.02 ± 0.01
PUFA/SFA	1.72 ± 0.03		
n6/n3	1.56 ± 0.01		

fw – fresh weight; dw – dry weight.
Palmitic acid (C16:0); Stearic acid (C18:0); Oleic acid (C18:1n9c); Linoleic acid (C18:2n6c); α-Linolenic acid (C18:3n3). Nineteen more fatty acids were also identified and quantified (total relative percentage 8.42%; data not shown).
SFA – saturated fatty acids; MUFA – monounsaturated fatty acids; PUFA – polyunsaturated fatty acids.

3.2. Analysis of phytochemicals in the herb and in its decoction and infusion

As *C. nobile* is mostly consumed as decoctions and infusions (aqueous extracts), a comparative study of phytochemicals present in the herb and in those preparations was performed.

Oxalic, quinic, malic, citric and fumaric acids were quantified in all the extracts of *C. nobile* (Table 2), malic acid being the most abundant organic acid. Infusion was the preparation with the highest content in organic acids (9.07 g/100 g dw), while the decoction showed the lowest amount (6.58 g/100 g dw). Some of the mentioned compounds have shown bioactive properties such as the capacity to chelate metal ions of citric acid by forming bonds between the metal and its carboxyl or hydroxyl groups. Citric acid is effective in retarding the oxidative deterioration of lipids in foods and is commonly added to vegetable oils after deodorization (Hraš, Halodin, Knez, & Bauman, 2000). Oxalic acid has a strong chelating ability with multivalent cations; nevertheless, it has been considered as an antinutrient due to the inhibitory effect on mineral bioavailability and its formative effect on calcium oxalate urinary stones (Kayashima & Katayama, 2002).

The main phenolic compounds found in *C. nobile* herbal material and in its decoction and infusion were flavonoids (flavonols and flavones), phenolic acids and derivatives (Tables 3 and 4). In general, all the preparations revealed the same chromatographic profile, exemplified in Fig. 1A for the herbal sample. Up to thirty-one phenolic compounds, including a phenolic acid, 11 hydroxycinnamic acid derivatives and 19 flavonoids were detected in the *C. nobile* preparations (Table 3).

Peak 3 was identified as protocatechuic acid by comparison of its UV spectrum and retention time with a commercial standard. Six hydroxycinnamic acid derivatives (peaks 1, 2, 5, 13, 22 and 24) showed UV spectra with maximum wavelengths around 320–330 nm and yielded fragment ions at *m/z* 191 (deprotonated quinic acid) and 179 (deprotonated caffeic acid) in their MS^2 mass spectra, which together with their pseudo molecular ions $[M-H]^-$ at *m/z* 353, 515 and 677, allowed their identification as quinic acid derivatives containing one, two or three caffeic acid moieties, respectively. Peak assignments of the different caffeoylquinic acid isomers were made using the recommended IUPAC numbering system (IUPAC, 1976) as also the hierarchical keys previously developed by Clifford, Johnston, Knight, and Kuhnert (2003) and Clifford, Knight, and Kuhnert (2005). The major peak (peak 5 $[M-H]^-$ at *m/z* 353) was positively identified as 5-*O*-caffeoylquinic acid by comparison with an authentic standard. Peak 1 ($[M-H]^-$ at *m/z* 353) was identified as 3-*O*-caffeoylquinic acid, yielding the base peak at *m/z* 191 and the ion at *m/z* 179 with an intensity >50% base peak. Similar fragmentation pattern was reported by Clifford et al. (2003, 2005) as characteristic to distinguish 3-acylchlorogenic acids. Peaks 2, 22, 24 ($[M-H]^-$ at *m/z* 515) could be assigned as dicaffeoylquinic acids. Peaks 22 and 24 were assigned to 3,4-*O*- and 3,5-*O*-dicaffeoylquinic acids, respectively, based on their elution order, fragmentation pattern and relative abundances (Clifford et al., 2003, 2005). MS^2 fragmentation of peak 22 yielded

Table 2
Organic acids composition of *Chamaemelum nobile* (mean ± SD).

Organic acid	Herb	Decoction	Infusion
Oxalic acid	2.02 ± 0.06^a	1.74 ± 0.21^b	1.99 ± 0.13^{ba}
Quinic acid	1.74 ± 0.13^b	1.40 ± 0.04^b	2.56 ± 0.17^a
Malic acid	3.02 ± 0.07^a	2.21 ± 0.19^b	3.06 ± 0.05^a
Citric acid	1.33 ± 0.01^a	1.23 ± 0.16^a	1.46 ± 0.24^a
Fumaric acid	0.02 ± 0.00^a	0.01 ± 0.00^b	0.01 ± 0.00^b
Total (g/100 g)	8.14 ± 0.28^b	6.58 ± 0.28^c	9.07 ± 0.01^a

In each row different letters mean significant differences ($p < 0.05$).

Table 3
Retention time (Rt), wavelengths of maximum absorption in the UV–vis region (λ_{max}), pseudomolecular and MS^2 fragment ions (in brackets, relative abundances) and tentative identification of the phenolic compounds of *Chamaemelum nobile*.

Peak	Rt (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification
1	5.11	326	353	191 (100), 179 (69), 161 (7), 135 (51)	3-*O*-Caffeolyquinic acid
2	5.65	320	515	353 (11), 341 (6), 323 (100), 191 (61), 179 (6), 161 (18), 135*	5-*O*-Caffeoylquinic acid-hexoside
3	6.15	262,294	153	109 (100)	Protocatechuic acid
4	6.44	326	469	307 (8), 179 (100),161 (37), 135 (78)	Caffeoyl-hexoside-methylglutarate
5	7.79	326	353	191 (100), 179 (11), 173 (9), 161 (28), 135 (8)	5-*O*-caffeolyquinic acid
6	9.62	312	453	291*, 163 (100), 145 (9), 119 (51)	*p*-Coumaroyl-hexoside-methylglutarate
7	10.48	332	593	593 (100), 473 (18), 383 (6), 353 (12)	Apigenin 6-*C*-glucose-8-*C*-glucose
8	11.05	316	453	163 (100), 145 (8),119 (42)	*p*-Coumaroyl–hexoside-metylglutarate
9	11.64	328	469	469 (100), 307*, 179 (6),161 (19), 135 (3)	Caffeoyl-hexoside-methylglutarate
10	16.19	320	445	445 (100), 427 (58), 265 (15), 179 (8)	Caffeic acid derivative
11	16.53	356	479	317 (100)	Myricetin 3-*O*-glucoside
12	17.15	344	621	487 (3), 351 (100), 269 (14)	Apigenin derivative
13	17.32	320	677	677 (100), 515 (28), 497 (16), 353 (65), 335 (23), 191 (12), 179 (8), 135 (3)	1,3,5-*O*-Tricaffeoylquinic acid
14	18.26	356	477	301 (100)	Quercetin 3-*O*-glucuronide
15	18.52	342	607	269 (100)	Apigenin *O*-glucuronylhexoside
16	18.71	350	579	579 (100), 417 (26), 285 (53)	Kaempferol *O*-pentosylhexoside
17	19.01	350	593	285 (100)	Luteolin *O*-rutinoside
18	19.12	344	607	545 (5), 337 (27), 269 (100)	Apigenin derivative
19	19.63	350	579	579 (100), 447 (4), 285 (16)	Luteolin *O*-pentosylhexoside
20	19.70	350	461	285 (100)	Luteolin *O*-glucuronide
21	20.06	344	593	593 (100), 447 (5), 285 (17)	Luteolin *O*-rhamnosylhexoside
22	20.11	332	515	515 (100), 353 (54), 335 (16),299 (3),203 (3),191 (16), 179 (27), 173 (44),135 (11)	3,4-*O*-Dicaffeoylquinic acid
23	21.14	350	447	285 (100)	Luteolin *O*-hexoside
24	21.75	328	515	353 (100), 335 (6), 191 (85), 179 (42), 173 (11), 135 (16)	3,5-*O*-Dicaffeoylquinic acid
25	23.59	370	549	505 (100), 301 (74)	Quercetin 7-*O*-malonylhexoside
26a	24.26	318sh,348	505	301 (100)	Quercetin *O*-acetylhexoside
26b	24.23	338	445	269 (100)	Apigenin *O*-glucuronide
27	24.52	338	649	605*, 587 (5), 515 (5), 427 (5), 379 (29), 361 (3), 311 (4), 269 (100)	Apigenin derivative
28	25.05	332	489	285 (100)	Luteolin *O*-acetylhexoside
29	25.27	334	445	269 (100)	Apigenin *O*-glucuronide
30	27.18	318sh,358	519	315 (100)	Isorhamnetin *O*-acetylhexoside

* abundance ⩽ 2; 26b-only found in decoction sample.

the formation of relatively intense signals corresponding "dehydrated" fragments at *m/z* 335 [caffeoylquinic acid-H–H_2O]$^-$ and *m/z* 173 [quinic acid-H–H_2O]$^-$, characteristic of 4-acyl-caffeoylquinic acids. Furthermore, according to Clifford et al. (2005), the intensity of signal at *m/z* 335 (16% of base peak) is more intense than in 4,5-*O*-dicaffeoylquinic (barely detectable, <5% of base peak). These observations allowed assigning peak 22 as 3,4-*O*-dicaffeoylquinic acid. The fragmentation pattern for 3,5-*O*-dicaffeoylquinic (peak 24) acid was similar to the one previously reported by Clifford et al. (2005). MS^2 base peak was at *m/z* 353, produced by the loss of one of the caffeoyl moieties [M–H-caffeoyl]$^-$, and subsequent fragmentation of this ion yielded the same fragments as a 5-caffeoylquinic acid at *m/z* 191, 179 and 135, although in this case with a comparatively more intense signal at *m/z* 179 [caffeic acid-H]$^-$ (<50% base peak).

Peak 2 showed the same pseudomolecular ion as peaks 22 and 24 but eluted much earlier than those dicaffeoylquinic acids and also than 5-*O*-caffeoylquinic acid. In addition to the fragments characteristic of a caffeoylquinic acid, the MS^2 spectrum of this compound also produced fragments at *m/z* 353 ([M–H-162]$^-$, loss of a hexose residue) and 341 ([M–H-174]$^-$, loss of a quinic acid moiety) and its "dehydrated" form at *m/z* 323. This fragmentation pattern pointed to a glycosylated chlorogenic acid, which could correspond to 1- or 5-caffeoylquinic-hexoside, according to the mass spectra characteristics and intensities of MS^2 fragment ions as reported by Clifford, Wu, Kirkpatrick, and Kuhnert (2007). The fact that 5-*O*-caffeoylquinic acid was the majority compound in the sample and 1-*O*-caffeoylquinic acid was not detected, permitted its tentative identification as 5-*O*-caffeoylquinic acid-hexoside, identity that was coherent with its early elution (greater polarity) compared with its parent aglycone.

Peak 13 was identified as tricaffeoylquinic acid according to its pseudomolecular ion [M–H]$^-$ at *m/z* 677 and diagnostic MS^2 fragments at *m/z* 515 (loss of the first caffeoyl), *m/z* 353 (loss of the second caffeoyl), *m/z* 191 (loss of the third caffeoyl to give quinic acid ion), *m/z* 179 (caffeic acid ion). The signal observed at *m/z* 497 can be interpreted by the loss of a water molecule from the ion at *m/z* 515. According to the relative intensities of different tricaffeoylquinic acid isomers reported by Lin and Harnly (2008), this peak could be assigned as 1,3,5-*O*-tricaffeoylquinic acid.

Peaks 4, 9 and 10, with UV spectra similar to caffeic acid with λ_{max} around 326 nm, were also assigned to caffeic acid derivatives. All of them presented an MS^2 fragment at *m/z* 179 ([caffeic acid-H]$^-$). Peaks 4 and 9 also presented a fragment at *m/z* 307 (−162 mu, loss of a hexose moiety), and the formation of the ion at *m/z* 179 could be produced by the loss of 162 + 128 mu (loss of hexose + methyl-glutarate residues), so that they were tentatively identified as caffeoyl-hexoside-methylglutarate. Although they could not be fully identified, these compounds could be attributed as derived from the *cis* and *trans* isomers of caffeic acid. The MS^2 analysis of peak 10 yielded signals at *m/z* 427 ([M–H_2O]$^-$) and *m/z* 265 ([M–18-162]$^-$) pointing out to the presence of a hexose, although no further conclusions could be made about its definite identity.

Table 4
Quantification of phenolic compounds in *Chamaemelum nobile* (mean ± SD).

Peak	Herb	Decoction	Infusion
1	0.07 ± 0.01	nd	0.08 ± 0.01
2	0.21 ± 0.01	0.04 ± 0.01	0.15 ± 0.02
3	0.04 ± 0.01	0.06 ± 0.01	0.10 ± 0.0
4	0.07 ± 0.01	nd	0.05 ± 0.00
5	1.70 ± 0.00	nd	1.52 ± 0.04
6	tr	nd	tr
7	0.04 ± 0.00	0.03 ± 0.00	0.01 ± 0.00
8	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00
9	0.01 ± 0.00	nd	0.01 ± 0.00
10	0.01 ± 0.00	nd	0.02 ± 0.00
11	0.01 ± 0.00	nd	0.01 ± 0.00
12	0.11 ± 0.01	0.17 ± 0.01	0.15 ± 0.01
13	0.17 ± 0.04	nd	0.13 ± 0.00
14	0.10 ± 0.00	nd	0.05 ± 0.01
15	0.06 ± 0.01	0.02 ± 0.00	0.02 ± 0.00
16	0.04 ± 0.01	0.02 ± 0.00	nd
17	0.42 ±0.03	nd	0.11 ± 0.00
18	0.15 ± 0.01	0.50 ± 0.05	0.07 ± 0.01
19	0.31 ± 0.07	0.18 ± 0.01	0.13 ± 0.00
20	0.19 ± 0.04	0.07 ± 0.01	0.16 ± 0.00
21	0.37 ± 0.02	0.12 ± 0.01	nd
22	0.02 ± 0.00	nd	0.01 ± 0.00
23	0.37 ± 0.06	0.06 ± 0.00	0.39 ± 0.02
24	0.37 ± 0.07	nd	0.06 ± 0.02
25	0.11 ± 0.01	0.01 ± 0.00	0.03 ± 0.00
26a	0.05 ± 0.01	nd	0.04 ± 0.01
26b	nd	0.06 ± 0.01	nd
27	0.88 ± 0.05	0.29 ± 0.03	0.76 ± 0.02
28	0.46 ± 0.07	nd	0.24 ± 0.00
29	0.12 ± 0.01	nd	0.10 ± 0.0
30	0.02 ± 0.00	nd	0.11 ± 0.00
Total flavonol derivatives (g/100 g)	0.34 ± 0.03[a]	0.02 ± 0.00[c]	0.13 ± 0.01[b]
Total flavone derivatives (g/100 g)	3.48 ± 0.25[a]	1.51 ± 0.02[c]	2.18 ± 0.03[b]
Total caffeoylquinic acids (g/100 g)	2.54 ± 0.13[a]	0.04 ± 0.01[c]	1.94 ± 0.07[b]
Total phenolic acid derivatives (g/100 g)	0.15 ± 0.02[b]	0.08 ± 0.01[c]	0.19 ± 0.00[a]
Total phenolic compounds (g/100 g)	6.51 ± 0.43[a]	1.65 ± 0.03[c]	4.44 ± 0.11[b]

In each row different letters mean significant differences ($p < 0.05$). tr – Traces; nd – not detected.

Peaks 6 and 8 ([M–H]$^-$ at *m/z* 453) presented a molecular weight 16 units lower than peaks 4 and 9 but with a similar fragmentation pattern, indicating that they could be the corresponding coumaroyl derivatives; this assumption was also supported by the formation of the MS^2 fragment ion at *m/z* 163 ([coumaric acid-H]$^-$). Thus, they were tentatively identified as two *p*-coumaroyl-hexoside-methylglutarate. Their later elution (lower polarity) compared with the caffeoyl counterparts (peaks 4 and 9) was also coherent with this identity; similarly, they could be speculated as the respective *cis* and *trans* isomers.

Flavones were the most abundant flavonoids present in the analysed samples (Table 4). Peaks 7, 12, 15, 18, 26b, 27 and 29 were identified as apigenin derivatives, according to their UV and mass spectra characteristics (Fig. 1B). Peak 7 presented a pseudo molecular ion [M–H]$^-$ at *m/z* 593, releasing three MS^2 fragments ions at *m/z* 473 and 383, corresponding to loss of 120 and 90 amu, characteristic of *C*-hexosyl flavones, and at *m/z* 353 that might correspond to the apigenin aglycone bearing some sugar residues [apigenin + 83 mu] (Ferreres, Silva, Andrade, Seabra, & Ferreira, 2003). The fact that no relevant fragment derived from the loss of a complete hexosyl residue (–162 mu) was detected, suggested that both sugars were *C*-attached, which allowed a tentative identification of the compound as apigenin 6-*C*-glucose-8-*C*-glucose. Peaks 15, 26b and 29 presented pseudo molecular ions [M–H]$^-$ at *m/z* 607 and 445 releasing a MS^2 fragment ion at *m/z* 269 ([M–176-162]$^-$ and [M–176]$^-$, corresponding to the loss of glucuronyl-hexoside and glucuronyl moieties, respectively), being tentatively identified as apigenin *O*-glucuronyl-hexoside (peak 15) and apigenin *O*-glucuronides (peaks 26b and 29).

Peaks 12, 18 and 27 presented pseudomolecular ions [M–H]$^-$ at *m/z* 621, 607 and 649, respectively, that release an MS^2 fragment at *m/z* 269 (apigenin). They all presented a similar fragmentation pattern, with a loss of 270 mu, and peaks 18 and 27 also have a fragment ion at [M–338]$^-$, that could correspond to glucuronyl-hexoside or feruloyl-hexoside [M–176-162]. Moreover, peak 27 presents a difference of 42 mu relatively to peak 18, that may be due to an acyl group. Peaks 12 and 18 also presented a difference of 14 mu that could correspond to a methyl group. However, the fragmentation patterns of these compounds did not allow us to conclude further about their chemical structure, but due to the UV spectra (Fig. 1B) and the fragmentation mentioned above they were associated to unknown apigenin derivatives.

Peaks 17, 19, 20, 21, 23 and 28 were identified as luteolin derivatives. Peaks 17, 20, 23 and 28 presented pseudomolecular ions [M–H]$^-$ at *m/z* 593, 461, 447 and 489 releasing a common MS^2 fragment at *m/z* 285 ([M–308]$^-$, [M–176]$^-$, [M–162]$^-$ and [M–42-162], associated to the loss of rutinosyl, glucuronyl, hexosyl and acetylhexoside moieties, respectively). They were tentatively identified as luteolin *O*-rutinoside, luteolin *O*-glucuronide, luteolin *O*-hexoside (the retention time is different from luteolin

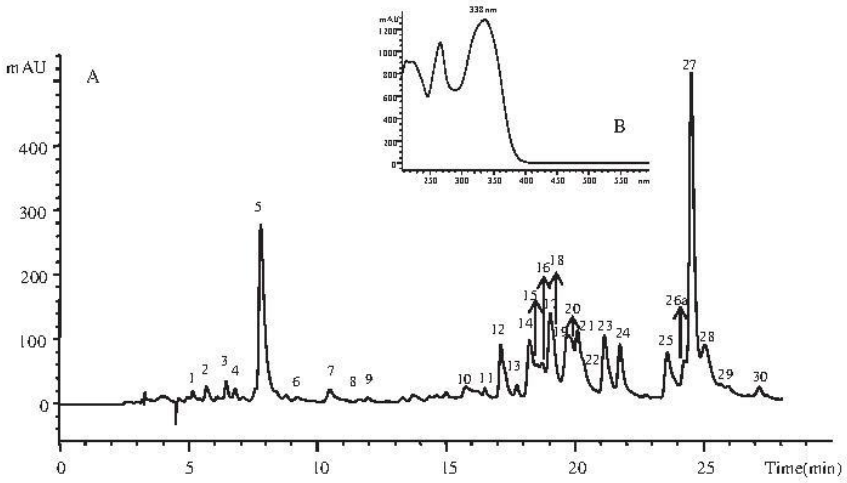

Fig. 1. HPLC chromatogram of the phenolic compounds of *Chamaemelum nobile* recorded at 370 nm (A) and UV spectra of peak 27 (B).

7-*O*-hexoside) and luteolin *O*-acetylhexoside. Peaks 19 and 21 showed pseudomolecular ions $[M-H]^-$ at *m/z* 579 and 593, both releasing two MS^2 fragments at *m/z* 447 ($[M-132]^-$ and $[M-146]^-$ loss of pentosyl and rhamnosyl moieties, respectively) and 285 ($[M-162]^-$, loss of a hexosyl moiety), being tentatively identified as luteolin *O*-pentosyl-hexoside and luteolin *O*-rhamnosyl-hexoside.

Flavonols (peaks 11, 14, 16, 25, 26a and 30) were also found in the studied samples (Tables 3 and 4). Peak 11 presented a pseudomolecular ion $[M-H]^-$ at *m/z* 479, releasing an MS^2 fragment at *m/z* 317 ($[M-H-162]^-$, loss of an hexosyl moiety), corresponding to myricetrin, which allowed a tentative identification of the compound as myricetin 3-*O*-glucoside, as confirmed by comparison with an authentic standard. Peak 16 showed a pseudomolecular ion $[M-H]^-$ at *m/z* 579, releasing two MS^2 fragments at *m/z* 417 ($[M-H-162]^-$, loss of a hexosyl moiety) and 285 (kaempferol; $[M-H-162-132]^-$, loss of a further pentosyl moiety), being tentatively identified as kaempferol pentosyl-glucoside the two glycosyl residues are assumed to constitute a disaccharide taking into account that they are lost successively and no alternatively, with no fragment corresponding to a kaempferol-pentoside being released.

Peaks 14, 25 and 26a corresponded to quercetin derivatives. Peak 14 presented a pseudomolecular ion $[M-H]^-$ at *m/z* 477, releasing an MS^2 fragment at *m/z* 301 ($[M-H-176]^-$, loss of a glucuronyl moiety); this compound was identified as quercetin 3-*O*-glucuronide, by comparison with a standard isolated in our laboratory (Dueñas et al., 2008). Peak 25 presented a pseudomolecular ion $[M-H]^-$ at *m/z* 549, releasing a MS^2 fragments at *m/z* 301 ($[M-H-162-86]^-$, loss of a malonylhexoside moiety). The UV–vis spectra have long been used for structural analysis of flavonoids. The typical flavonoid spectrum consists of two maxima in the range 240–285 nm (Band II), and 300–550 nm (Band I), which is more specific and useful for obtaining information regarding identification. The position and relative intensities of these maxima yield information on the nature of the flavonoid and its hydroxylation and substitution patterns (Santos-Buelga, Garcia-Viguera, & Tomas-Barberan, 2003). It is known that the introduction of a glycoside on the hydroxyls at positions 7, 3′ or 4′ has no effect on wavelength maxima or the spectrum shape in relation to the aglycone. Thus, quercetin 7-*O*-glycosides would have λ_{max} in Band I around 370 nm, while quercetin 3 *O*-glycosides are hypsochromically shifted to around 354 nm. Since peak 25 presented λ_{max} at 370 nm it was tentatively identified as quercetin 7-*O*-malonylhexoside.

Peak 26a presented a pseudomolecular ion $[M-H]^-$ at *m/z* 505 releasing an MS^2 fragment at *m/z* 301 (quercetin; $[M-H-42-162]^-$, loss of an acetylhexoside moiety), and was tentatively identified as quercetin *O*-acetylhexoside. Peak 30 presented a pseudomolecular ion $[M-H]^-$ at *m/z* 519 releasing a MS^2 fragment at *m/z* 315 (isorhmanetin; $[M-H-42-162]^-$, loss of an acetylhexoside moiety), so that it was tentatively identified as isorhamnetin *O*-acetylhexoside.

The amounts of the phenolic compounds found varied among the different preparations and some compounds disappeared, mostly in decoctions (Table 4). Peak 26a, quercetin *O*-acetylhexoside that appeared in the plant material and infusion preparation, was not present in the decoction preparation, being detected at the same retention time another compound (peak 26b) associated to an apigenin glucuronide. Tschan et al. (1996) and Carnat et al. (2004) reported in *C. nobile* the presence of chamaemeloside (i.e., apigenin 7-glucoside-6″-(3‴-hydroxy-3‴-methyl-glutarate), but this compound was not detected in the sample of roman chamomile studied herein. Carnat et al. (2004) did not report in their study the presence in roman chamomile of some flavonols described in the present work, such as the quercetin, kaempferol and isorhamnetin derivatives, but they only report flavones and phenolic acids also detected in this study.

3.3. Evaluation of bioactivity

The antioxidant properties were evaluated by four different tests as there is no universal method that can measure the antioxidant capacity of all samples accurately and quantitatively: DPPH radical scavenging capacity, reducing power and inhibition of lipid peroxidation using β-carotene–linoleate model system in liposomes and TBARS assay in brain homogenates. As it can be observed in Table 5, herbal sample gave the highest β-carotene bleaching activity and lipid peroxidation inhibition (lowest EC_{50} values, Table 5) which can be related to its higher content in phenolic compounds (Table 4), while infusion showed the highest DPPH scavenging activity (Table 5) which may be related to their higher levels in organic acids (Table 2). Both samples showed similar reducing power (Table 5). The decoction presented the lowest antioxidant properties, probably due to its lower content in phenolic compounds and organic acids.

The effects of *C. nobile* extract, decoction and infusion on the growth of five human tumour cell lines (MCF-7, NCI-H460, HCT-15, HeLa and HepG2), represented as the concentrations that caused 50% of cell growth inhibition (GI_{50}), are also summarised in Table 5. The plant material extract was more potent than the infusion sample in all the tested cell lines, presenting GI_{50} values that ranged from 82.52 to 168.40 μg/ml for the MCF-7 and HepG2 cells, respectively. Decoction preparation had no antitumour effects at the maximal concentration used (400 μg/ml), which could

Table 5
Antioxidant and antitumour activities, and hepatotoxicity of *Chamaemelum nobile* (mean ± SD).

	Herb	Decoction	Infusion	Standard[a]
Antioxidant activity (EC_{50} values, μg/ml)				
DPPH scavenging activity	621.64 ± 6.84[b]	1477.30 ± 71.99[a]	408.46 ± 11.34[c]	43.03 ± 1.71
Reducing power	294.90 ± 10.97[b]	532.62 ± 8.75[a]	258.89 ± 3.36[b]	29.62 ± 3.15
β-Carotene bleaching inhibition	443.32 ± 1.47[c]	680.54 ± 69.06[b]	1219.64 ± 31.56[a]	2.63 ± 0.14
TBARS inhibition	82.33 ± 11.53[c]	268.55 ± 5.32[a]	120.41 ± 4.24[b]	3.73 ± 1.90
Antitumour activity (GI_{50} values, μg/ml)				
MCF-7 (breast carcinoma)	82.52 ± 4.57[b]	>400	247.95 ± 5.56[a]	0.91 ± 0.04
NCI-H460 (non-small cell lung cancer)	82.75 ± 8.14[b]	>400	226.65 ± 7.42[a]	1.42 ± 0.00
HCT-15 (colon carcinoma)	91.23 ± 3.13[b]	>400	150.24 ± 5.47[a]	1.91 ± 0.06
HeLa (cervical carcinoma)	85.01 ± 6.39[b]	>400	233.06 ± 7.36[a]	1.14 ± 0.21
HepG2 (hepatocellular carcinoma)	168.40 ± 2.23[b]	>400	250.30 ± 0.55[a]	3.22 ± 0.67
Hepatotoxicity (GI_{50} value, μg/ml)				
PLP2	>400	>400	>400	2.06 ± 0.03

[a] Trolox and ellipticine for antioxidant and antitumour activity assays, respectively. EC_{50} values correspond to the sample concentration achieving 50% of antioxidant activity or 0.5 of absorbance in reducing power assay. GI_{50} values correspond to the sample concentration achieving 50% of growth inhibition in human tumour cell lines or in liver primary culture PLP2. In each row different letters mean significant differences ($p < 0.05$).

indicate that these effects might be related to compounds (including phenolic compounds) that are not extracted or affected by the decoction. Nevertheless, none of the *C. nobile* preparation showed hepatotoxicity in the porcine liver primary cell culture (non-tumour cells; PLP2) (Table 5).

Overall, *C. nobile* is an equilibrated valuable species rich in carbohydrates and proteins, and poor in fat, providing tocopherols, carotenoids and essential fatty acids (C18:2n6 and C18:3n3). Moreover, the herb and its infusion are a source of phenolic compounds and organic acids that showed antioxidant and antitumour activities, without hepatotoxicity. Some bioactive compounds are affected by in *C. nobile* decoction, leading to a lower antioxidant potential and absence of antitumour potential. The plant bioactivity could be explored in the medicine, food, and cosmetic industries.

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References

Abreu, R. M. V., Ferreira, I. C. F. R., Calhelha, R. C., Lima, R. T., Vasconcelos, M. H., Adega, F., et al. (2011). Anti-hepatocellular carcinoma activity using human HepG2 cells and hepatotoxicity of 6-substituted methyl 3-aminothieno[3,2-*b*]pyridine-2-carboxylate derivatives: In vitro evaluation, cell cycle analysis and QSAR studies. *European Journal of Medicinal Chemistry, 46*, 5800–5806.

AOAC. (1995). *Official methods of analysis* (16th ed.). Arlington VA, USA: Association of Official Analytical Chemists.

Barros, L., Dueñas, M., Dias, M. I., Sousa, M. J., Santos-Buelga, C., & Ferreira, I. C. F. R. (2012). Phenolic profiles of in vivo and *in vitro* grown *Coriandrum sativum* L. *Food Chemistry, 132*, 841–848.

Barros, L., Pereira, C., & Ferreira, I.C.F.R. (2012). Optimized analysis of organic acids in edible mushrooms from Portugal by ultra fast liquid chromatography and photodiode array detection. *Food Analytical Methods*, in press, http://dx.doi.org/10.1007/s12161-012-9443-1.

Cao, Y. H., & Cao, R. H. (1999). Angiogenesis inhibited by drinking tea. *Nature, 398*(6726), 381.

Carnat, A., Carnat, A. P., Fraisse, D., Ricoux, L., & Lamaison, J. L. (2004). The aromatic and polyphenolic composition of Roman camomile tea. *Fitoterapia, 75*, 32–38.

Clifford, M. N., Johnston, K. L., Knight, S., & Kuhnert, N. A. (2003). A hierarchical scheme for LC–MSn identification of chlorogenic acids. *Journal of Agricultural and Food Chemistry, 51*, 2900–2911.

Clifford, M. N., Knight, S., & Kuhnert, N. A. (2005). Discriminating between the six isomers of dicaffeoylquinic acid by LC–MSn. *Journal of Agricultural and Food Chemistry, 53*, 3821–3832.

Clifford, M. N., Wu, W., Kirkpatrick, J., & Kuhnert, N. A. (2007). Profiling the chlorogenic acids and other caffeic acid derivatives of herbal chrysanthemum by HPLC–MSn. *Journal of Agricultural and Food Chemistry, 55*, 929–936.

Dueñas, M., Chornet, H. M., Pérez-Alonso, J. J., Paola-Naranjo, R. D., González-Paramás, A. M., & Santos-Buelga, C. (2008). Preparation of quercetin glucuronides and characterization by HPLC–DAD–ESI/MS. *European Food Research and Technology, 227*, 1069–1076.

Ferreres, F., Silva, B. M., Andrade, P. B., Seabra, R. M., & Ferreira, M. A. (2003). Approach to the study of C-glycosyl flavones by ion trap HPLC–PAD–ESI/MS/MS: Application to seeds of quince (*Cydonia oblonga*). *Phytochemical Analysis, 14*, 352–359.

Galleano, M., Verstraenten, S. V., Oteiza, P. I., & Fraga, C. G. (2010). Antioxidant actions of flavonoids: Thermodynamic and kinetic analysis. *Archives of Biochemistry and Biophysics, 501*, 23–30.

Grassi, D., Desideri, G., Croce, G., Tiberti, S., Aggio, A., & Ferri, C. (2009). Flavonoids, vascular function and cardiovascular protection. *Current Pharmaceutical Design, 15*, 1072–1084.

Guil, J. L., Torija, M. E., Giménez, J. J., & Rodriguez, I. (1996). Identification of fatty acids in edible wild plants by gas chromatography. *Journal of Chromatography A, 719*, 229–235.

Hraš, A. R., Halodin, M., Knez, Z., & Bauman, D. (2000). Comparison of antioxidative and synergistic effects of rosemary extract with α-tocopherol, ascorbyl palmitate and citric acid in sunflower oil. *Food Chemistry, 71*, 229–233.

Iupac, K. (1976). Nomenclature of cyclitols. *Biochemistry Journal, 153*, 23–31.

Kandaswami, C., Lee, L. T., Lee, P. P., Hwang, J. J., Ke, F. C., Huang, Y. T., et al. (2005). The antitumor activities of flavonoids. *In Vivo, 19*, 895–909.

Kayashima, T., & Katayama, T. (2002). Oxalic acid is available as a natural antioxidant in some systems. *Biochimica et Biophysica Acta, 1573*, 1–3.

Lin, L. Z., & Harnly, J. M. (2008). Identification of hydroxycinnamoylquinic acids of arnica flowers and burdock roots using a standardized LC–DAD–ESI/MS profiling method. *Journal of Agricultural and Food Chemistry, 56*, 10105–10114.

López-Lázaro, M. (2002). Flavonoids as anticancer agents: Structure–activity relationship study. *Current Medicinal Chemistry Anti-Cancer Agents, 2*, 691–714.

Ma, C., Winsor, L., & Daneshtalab, M. (2007). Quantification of spiroether isomers an herniarin of different parts of *Matricaria matricarioides* and flowers of *Chamaemelum nobile*. *Phytochemical Analysis, 18*, 42–49.

Mladěnka, P., Zatloukalová, L., Filipský, T., & Hrdina, R. (2010). Cardiovascular effects of flavonoids are not caused only by direct antioxidant activity. *Free Radical Biology & Medicine, 49*, 963–975.

Nagata, M., & Yamashita, I. (1992). Simple method for simultaneous determination of chlorophyll and carotenoids in tomato fruit. *Nippon Shokuhin Kogyo Gakkaish, 39*, 925–928.

Pereira, E., Barros, L., Martins, A., & Ferreira, I. C. F. R. (2012). Towards chemical and nutritional inventory of Portuguese wild edible mushrooms in different habitats. *Food Chemistry, 130*, 394–403.

Santos-Buelga, C., Garcia-Viguera, C., & Tomas-Barberan, A. (2003). On-line identification of flavonoids by HPLC coupled to diode array detection. In *Methods in Polyphenol Analysis* (pp. 92–128). Cambridge: Royal Society of Canada.

Schroeter, H., Heiss, C., Balzer, J., Kleinbongard, P., Keen, C. L., Hollenberg, N. K., et al. (2006). (−)-Epicatechin mediates beneficial effects of flavonol-rich cocoa on vascular function in humans. *Proceedings of the National Academy of Sciences USA, 103*, 1024–1029.

Srivastava, J. K., Shankar, E., & Gupta, S. (2010). Chamomile: A herbal medicine of the past with a bright future (Review). *Molecular Medicine Reports, 3*, 895–901.

Tschan, G. M., Konig, G. M., Wright, A. D., & Stichert, O. (1996). Chamaemeloside, a new flavonoid glycoside from *Chamaemelum nobile*. *Phytochemistry, 41*, 643–646.

7.2 “Wild Roman chamomile extracts and phenolic compounds: enzymatic assays and molecular modelling studies with VEGFR-2 tyrosine Kinase”

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Wild Roman chamomile extracts and phenolic compounds: enzymatic assays and molecular modelling studies with VEGFR-2 tyrosine kinase

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Angiogenesis is a process by which new blood vessels are formed from the pre-existing vasculature, and it is a key process that leads to tumour development. Some studies have recognized phenolic compounds as chemopreventive agents; flavonoids, in particular, seem to suppress the growth of tumor cells modifying the cell cycle. Herein, the antiangiogenic activity of Roman chamomile (*Chamaemelum nobile* L.) extracts (methanolic extract and infusion) and the main phenolic compounds present (apigenin, apigenin-7-*O*-glucoside, caffeic acid, chlorogenic acid, luteolin, and luteolin-7-*O*-glucoside) was evaluated through enzymatic assays using the tyrosine kinase intracellular domain of the Vascular Endothelium Growth Factor Receptor-2 (VEGFR-2), which is a transmembrane receptor expressed fundamentally in endothelial cells involved in angiogenesis, and molecular modelling studies. The methanolic extract showed a lower IC_{50} value (concentration that provided 50% of VEGFR-2 inhibition) than the infusion, 269 and 301 μg mL^{-1}, respectively. Regarding phenolic compounds, luteolin and apigenin showed the highest capacity to inhibit the phosphorylation of VEGFR-2, leading us to believe that these compounds are involved in the activity revealed by the methanolic extract.

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1. Introduction

Angiogenesis is a process by which new blood vessels are formed from the pre-existing vasculature, developing a hemovascular network.[1] It is tightly controlled by a balance of angiogenesis factors and inhibitors, occurring in the embryonic development, wound healing and the female reproductive cycle. Angiogenic diseases result from new blood vessels growing either excessively (*e.g.* cancer, diabetic retinopathy and psoriasis) or insufficiently (*e.g.* chronic wounds and ischaemic heart disease).[1,2]

During angiogenesis, endothelial cells degrade the basement membrane, migrate into the surrounding intercellular matrix, proliferate to form new blood vessels, and differentiate into contiguous tubular sprouts, which subsequently form functional capillary loops. Such cellular events are mediated by various intracellular signal transduction pathways.[3,4] Angiogenesis happens in the body all the time. It occurs through a so-called angiogenesis "cascade" which involves a series of biochemical steps by which cells make and secrete molecules that initiate the growth of capillaries. After the process is over, certain other molecular "factors" turn off the angiogenesis process. Cancer cells use this normal process for another purpose, creating an imbalance of angiogenesis activators that overrides the inhibitors and gives the nearby tumour ready access to a blood supply.[5] This explains why angiogenesis is essential for the growth, progression, and metastasis of solid tumours.[6]

In the mentioned pathophysiological processes, excessive angiogenesis occurs when diseased cells produce abnormally large amounts of angiogenesis factors [*e.g.* vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF)-2 and hepatocyte growth factor], overcoming the effects of natural angiogenesis inhibitors (*e.g.* angiostatin, endostatin and thrombospondin).[1]

VEGF is a secreted growth factor by tumor cells that plays a critical role in angiogenesis; low oxygen tension dramatically induces the expression of this major angiogenic factor.[7] Its biological effects are mediated by two receptor tyrosine kinases namely VEGFR-1 (fms-like tyrosine kinase, Flt-1) and VEGFR-2 (kinase-insert domain-containing receptor, KDR), which differ considerably in their signalling characteristics.[8,9] Although increasing evidence indicates that angiogenesis is a highly sophisticated and coordinated process, the activation of

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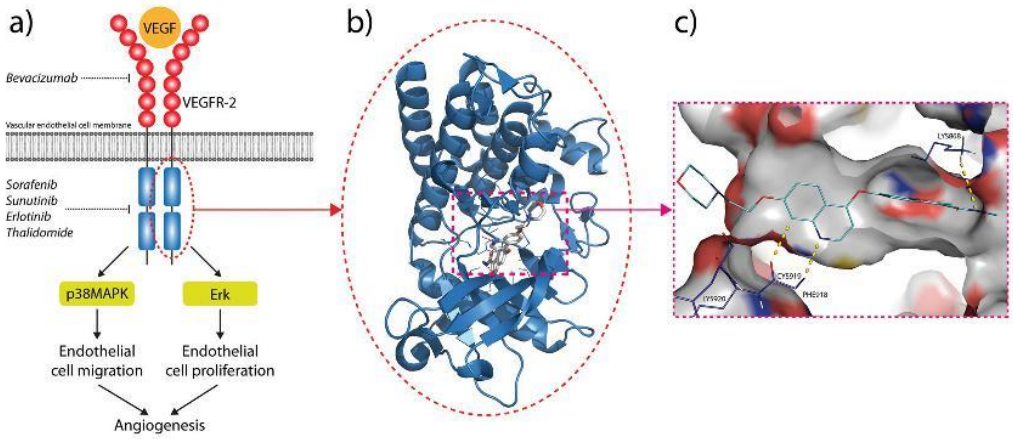

Fig. 1 (a) Main angiogenesis signaling pathways mediated by VEGFR-2; (b) X-ray crystal structure of the VEGFR-2 intracellular tyrosine kinase domain (PDB: 2XIR), co-crystallized with a TKI; (c) detailed representation of the ATP binding pocket and adjacent binding pockets showing the main interactions between VEGF-2 and the TKI (PDB: 2XIR).

the VEGF/VEGFR pathway remains the key modulator of angiogenesis.[10] Furthermore, VEGF is the leading angiogenic factor involved in tumoral angiogenesis.[7,9]

Of the primary receptors, VEGFR-2 is thought to mediate the majority of tumor angiogenic effects (Fig. 1a). Current clinical treatments against tumor antiangiogenesis that target VEGFR-2 include: monoclonal antibodies (*e.g.* bevacizumab) that target the VEGFR-2 extracellular VEGF binding domain and small tyrosine kinase inhibitors (TKIs) that target the VEGFR-2 intracellular tyrosine kinase domain (Fig. 1b). TKIs act by binding to the ATP binding pocket and to the adjacent pockets thus preventing the phosphorylation of this intracellular domain (*e.g.*, sunitinib, sorafenib, ZD6474, erlotinib or thalidomide) and blocking the angiogenic signaling pathway (Fig. 1c), lowering blood tumoral irrigation, and improving chemotherapy distribution.[9]

Several polyphenolic compounds are recognized as cancer chemopreventive agents. Flavonoids are especially well known to suppress tumor cell growth *via* cell-cycle arrest and by the induction of apoptosis in several tumor cell lines.[11,12] Moreover, flavonoids namely genistein inhibit endothelial cell cultures on collagen gels.[13] The antiangiogenic effect of apigenin on tumor cells was also reported and related to a reduction in the expression of VEGF.[12]

Other plant-derived anticancer drugs (*e.g.* Taxol®, camptothecin and combretastatin) proved to be antiangiogenic. In traditional Chinese medicine, many herbs are used in the treatment of angiogenic diseases such as chronic wounds and rheumatoid arthritis.[1] Furthermore, it has been reported that drinking green tea could inhibit VEGF-induced angiogenesis *in vivo*.[5]

In a previous study, we reported the antitumor activity of Roman chamomile (*Chamaemelum nobile* L.) methanolic extract and infusion in five different human tumour cells (non-small cell lung cancer, breast, colon, cervical and hepatocellular carcinomas). Furthermore, flavonoids such as flavonols and flavones, phenolic acids and derivatives were found in this wild herb.[14] In the present work, the antiangiogenic activity of Roman chamomile (*Chamaemelum nobile* L.) extracts (methanolic extract and infusion) and main phenolic compounds (apigenin, apigenin-7-*O*-glucoside, caffeic acid, chlorogenic acid, luteolin, and luteolin-7-*O*-glucoside) were evaluated through enzymatic assays using the tyrosine kinase intracellular domain of VEGFR-2. To better understand the inhibition phosphorylation mechanism of the tyrosine kinase receptor by luteolin, apigenin and apigenin-7-*O*-glucoside, docking studies were performed.

2. Materials and methods

2.1 Biological material and sample preparation

C. nobile was gathered during the flowering season (June–July 2010) from wild populations located in grasslands in Bragança (Trás-os-Montes, Northeastern Portugal). Samples consist of pieces of about 8 cm, corresponding to terminal soft leafy stems and inflorescences with flowers fully open and functional, picked up in plants randomly selected in a meadow of about a hectare. The plant material was put together in a single sample for analysis. Voucher specimens are deposited in the Herbarium of the Escola Superior Agrária de Bragança (BRESA). The sample was lyophilized (FreeZone 4.5, Labconco, Kansas, USA), reduced to a fine dried powder (20 mesh) and mixed to obtain a homogenate sample.

A methanolic extract was prepared from the lyophilized plant material. The sample (1 g) was extracted by stirring with 25 mL of methanol (25 °C at 150 rpm) for 1 h and subsequently filtered through Whatman No. 4 paper. The residue was then extracted with 25 mL of methanol (25 °C at 150 rpm) for 1 h. The combined methanolic extracts were evaporated at 40 °C (rotary evaporator Büchi R-210) to dryness and re-dissolved in DMSO to a final concentration of 400 μg mL^{-1}.

An infusion was also prepared from the lyophilized plant material. The sample (1 g) was added to 200 mL of boiling distilled water and left to stand at room temperature for 5 min, and then filtered under reduced pressure. The obtained infusion was frozen, lyophilized and re-dissolved in DMSO to a final concentration of 400 μg mL^{-1}.

2.2. Phenolic compounds

Apigenin, apigenin-7-*O*-glucoside, caffeic acid, chlorogenic acid, luteolin, and luteolin-7-*O*-glucoside were from Extrasynthese (Genay, France). Each phenolic compound was dissolved in DMSO to a final concentration of 40 μg mL^{-1}.

2.3. VEGFR-2 enzymatic inhibition assay

C. nobile methanolic extract and infusion, and the pure phenolic compounds were assessed for VEGFR-2 inhibition activity using the Z′-LYTE-Tyr1 Peptide assay kit (Invitrogen, Cat. PV3190) according to the procedures recommended by the manufacturer.[15] Briefly, assays were performed in a total of 20 μL in 384-well plates using fluorescence resonance energy transfer technology. A Tyr1 substrate (coumarin-fluorescein double-labeled peptide) at 1 μM was incubated for 1 h with 4 μg mL^{-1} VEGFR-2, 50 μM ATP and the *C. nobile* methanolic extract/infusion (400 at 6.25 μg mL^{-1}) or the pure phenolic compounds (40 at 0.04 μg mL^{-1}) at room temperature in 50 mM Hepes/NaOH (pH 7.5), 10 mM $MgCl_2$, 2 mM $MnCl_2$, 2.5 mM DTT, 0.1 mM orthovanadate, and 0.01% bovine serum albumin. The wells were incubated at 25 °C for 1 h and 5 μL development reagents were added to each well. After a second incubation of 1 h a stop reagent was added to each well. Using a Biotek FLX800 micro-plate the fluorescence was read at 445 nm and 520 nm (excitation 400 nm), and Gen5™ Software was used for data analysis. Genistein (Extrasynthese, Genay, France) was used as positive control.

The assays were performed in triplicate and the results were expressed as mean values ± standard deviation (SD). The results were analyzed using a Student's *t*-test with $\alpha = 0.05$, to determine the significant difference among the two extracts. For the phenolic compounds, the analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's HSD test with $\alpha = 0.05$. These treatments were carried out using the SPSS v. 22.0 program.

2.4 Docking simulations using AutoDockVina

The 2D structure of the compounds apigenin, apigenin-7-*O*-glucoside, luteolin and luteolin-7-*O*-glucoside was constructed using the ACD/ChemSketch Freeware 12.0 software. Open Babel[16] was used to convert compounds from 2D to 3D and they were saved in the pdb format.

A VEGFR-2 crystal structure (PDB: 2XIR) was extracted from the Protein Data Bank (PDB) (http://www.rcsb.org). The co-crystallized ligand was extracted from the PDB file, and AutoDockTools[17] was used to assign polar hydrogens and Gasteiger charges to the compounds and VEGFR-2 protein. All structures were saved in the PDBQT file format required to use AutoDockVina.[18] AutodockVina was used to perform docking in an area of 30 Å × 30 Å × 30 Å, centered on the co-crystallized ligand. The docking simulations were performed on a cluster of 6 AMD Opteron 6128 8 core 2.0 GHz by using MOLA software.[19] All figures with structure representations were prepared using PyMOL (The PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC). Available at: (http://www.pymol.org/). Accessed on 03 September, 2012.

2.5 Molecular dynamics simulation

The protein preparation wizard from Maestro (Schrodinger, LLC, Portland, OR) was used to prepare ligand/VEGFR-2 complexes and then used to perform explicit solvent molecular dynamics (MD) simulations. The parallelized Desmond Molecular Dynamics System v2.2 (D. E. Shaw Research, New York, NY) and associated analysis tools, available within the Schrodinger suite (Schrodinger, LLC, Portland, OR), were used for this purpose. The protocol used was described by Mukherjee *et al.*[20]

3. Results and discussion

According to previous studies of the authors, Roman chamomile is an equilibrated valuable species rich in carbohydrates and proteins, and poor in fat, providing tocopherols, carotenoids and essential fatty acids (C18:2n6 and C18:3n3). Moreover, the herb and its infusion are a source of phenolic compounds and organic acids with high bioactive potential.[14] Herein, methanolic extract, infusion and phenolic compounds of Roman chamomile were evaluated for their ability to interact with the VEGFR-2 kinase domain, using an enzymatic (fluorescence resonance energy transfer) FRET-based assay. The results are shown in Table 1.

The methanolic extract showed a lower IC_{50} value than the infusion, 269 and 301 μg mL^{-1}, respectively. These results are in agreement with the higher phenolic compound amount, antioxidant and antitumor activities also previously reported for the methanolic extract.[14]

Table 1 VEGFR-2 inhibition activity of *Chamaemelum nobile* extracts and phenolic compounds (mean ± SD)

Chamaemelum nobile	VEGFR-2 IC_{50}, μg mL^{-1}
Methanolic extract	269.26 ± 8.74
Infusion	301.09 ± 13.07
Student's *t* test; *p*-value	<0.001
Phenolic compound	VEGFR-2 IC_{50}, μg mL^{-1}
Luteolin	0.60 ± 0.03^{c*}
Apigenin	1.29 ± 0.07^{b}
Apigenin-7-*O*-glucoside	19.21 ± 1.58^{a}
Luteolin-7-*O*-glucoside	>40
Caffeic acid	>40
Chlorogenic acid	>40
Genistein	1.04 ± 0.06

IC_{50}-concentration that provided 50% of VEGFR-2 inhibition. *Different letters mean significant differences between compounds ($p < 0.05$).

Regarding individual molecules, apigenin, apigenin-7-*O*-glucoside, caffeic acid, chlorogenic acid, luteolin and luteolin-7-*O*-glucoside were chosen because these compounds were the ones used to quantify all the phenolic compounds identified in Roman chamomile.[14] Phenolic acids (caffeic and chlorogenic acids) and luteolin-7-*O*-glucoside did not show the VEGFR-2 inhibition activity (IC_{50} values higher than 40 μg mL^{-1}), whereas apigenin-7-*O*-glucoside showed VEGFR-2 inhibition activity with the IC_{50} value = 19.21 μg mL^{-1}. A drastic increase in the VEGFR-2 inhibition activity was observed for the corresponding aglycones (compounds without the glycosyl group) of the mentioned flavonoids: luteolin and apigenin (IC_{50} values = 0.60 and 1.29 μg mL^{-1}, respectively). The active concentrations, corresponding to the last IC_{50} values, are easily provided by the Roman chamomile infusion, which contains 8.42 μg mL^{-1} and 9.28 μg mL^{-1} of luteolin and apigenin derivatives (compounds with glycosyl groups: luteolin-7-*O*-glucoside and apigenin-7-*O*-glucoside), respectively (values calculated from the ones reported previously by the authors and taking into account the extraction yields).[14] It should be highlighted that the methanolic extract prepared from the herb would provide even higher amounts of those derivatives (21.31 and 13.50 μg mL^{-1}, respectively[14]).

The possible VEGFR-2 inhibition mechanism of luteolin, apigenin and apigenin-7-*O*-glucoside (Fig. 2) was predicted using docking tools. A careful analysis of the predicted docking poses showed that apigenin and luteolin probably interact with the VEGFR-2 ATP binding site with a similar binding pose, stabilized by three predicted hydrogen bonds (Fig. 3): one H-bond between the CYS919 backbone and the carbonyl group at position 3 of the benzopyrone moiety; a second H-bond between the CYS919 backbone and the hydroxyl group at position 5 of the benzopyrone moiety; and a third H-bond between the amino group of the LYS868 side chain and the hydroxyl group at position 4 of the benzene ring. The higher VEGFR-2 inhibition capacity of luteolin compared to apigenin can probably be explained by the better occupation of the ATP binding site, accomplished by the lutein extra hydroxyl group occupation of a small pocket located inside the structure shown in Fig. 3. Furthermore, comparing the docking poses of apigenin and apigenin-7-*O*-glucoside, it was possible to observe that the presence of the glucoside moiety shifts the compound slightly away from the ATP binding site. This shift probably weakens the described H-bonds, explaining the lower VEGFR-2 inhibition capacity of apigenin-7-*O*-glucoside.

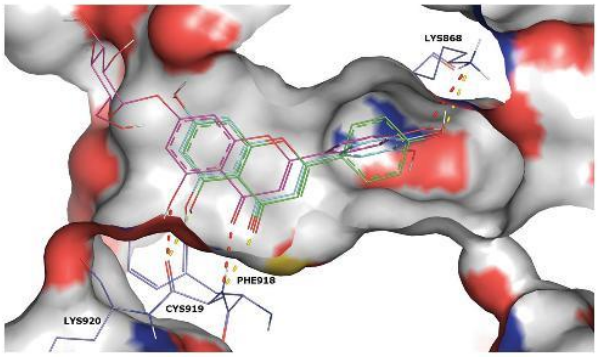

Fig. 3 Surface representation of the VEGFR-2 ATP binding site docked with apigenin (green line), luteolin (blue line) and apigenin-7-*O*-glucoside (magenta line). Apigenin and luteolin hydrogen bonds are represented as yellow dashes, and apigenin-7-*O*-glucoside hydrogen bonds as red dashes.

Moreover, the inability of AutodockVina to predict the binding pose of luteolin-7-*O*-glucoside similar to luteolin, apigenin and apigenin-7-*O*-glucoside seems to indicate that luteolin-7-*O*-glucoside probably cannot interact with the ATP binding site. This was experimentally proved by the high IC_{50} value obtained in the enzymatic assay (>100 μM).

MD (Molecular Dynamics) simulations were performed using the most active compounds, luteolin and apigenin, to verify whether both the predicted docking poses remain stable in a more physiologically relevant setting. The docking poses of both complexes were the starting points for 5 ns MD simulations, and the overall stability of each MD simulation was evaluated by plotting the receptor backbone (VEGFR-2) and ligands' RMSD (Root Mean Square Deviation) as a function of time (Fig. 4).

After small adjustments in the first ns of the MD simulation, both apigenin and luteolin structures remained stable during the MD simulation with an average RMSD of 0.37 and 0.57 Å, respectively (Fig. 4). This is an indication that the predicted docking pose is reliable and is probably close to the experimental VEGFR-2 binding pose. In both MD simulations, the RMSD values for the VEGFR-2 backbone structure were also analyzed and it was observed that, after a normal adjustment of around 2 ns, the RMSD values also remained stable during rest of the MD simulation. This is the expected MD simulation behavior of the protein backbone indicating that the VEGFR-2 structure used is suitable for this type of molecular modeling study.

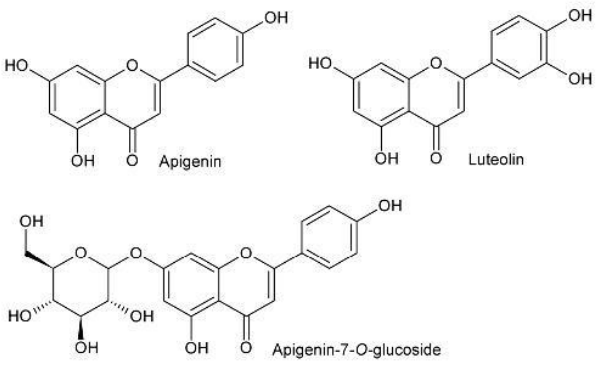

Fig. 2 Chemical structures of luteolin, apigenin and apigenin-7-*O*-glucoside.

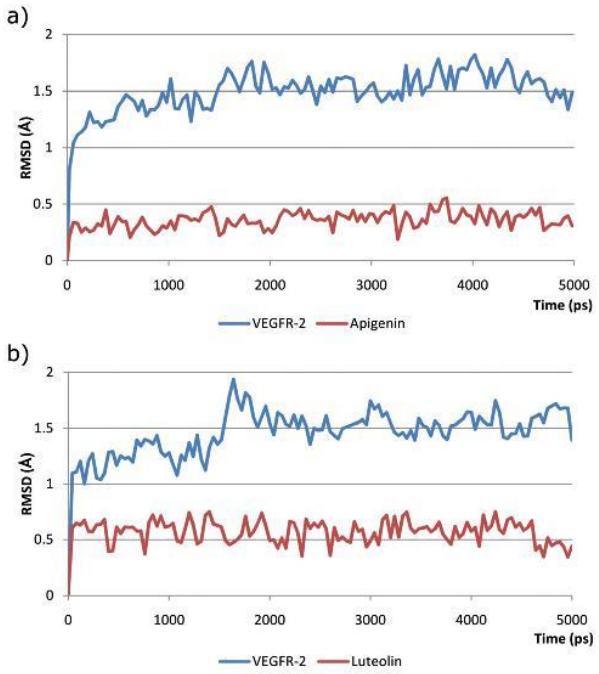

Fig. 4 RMSD values obtained during the 5 ns MD simulation timeframe for: (a) VEGFR-2/apigenin and (b) VEGFR-2/luteolin complexes.

In general the MD simulations performed gave us further assurance that the predicted docking pose probably corresponds closely to the experimental binding pose although this can only be completely established by the elucidation of the VEGFR-2/apigenin or VEGFR2/luteolin complex structures, usually performed by X-ray crystallography.

The antiangiogenic effect of apigenin on tumor cells has already been reported but related to the reduction in the expression of VEGF[12] and not to the inhibition of VEGFR activity, so it is demonstrated in the present work. Regarding luteolin, as far as we know this is the first report on antiangiogenic activity, and only its anticarcinogenic effects mainly by induction of apoptosis and cell cycle arrest by the action on critical molecular targets for cell survival such as p53, p21, cyclin dependent kinases and caspases in liver[21] and non-small cell lung[22] cancer cells are reported.

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References

1 T.-P. Fan, J.-C. Yeh, K. H. Leung, P. Y. K. Yue and R. N. S. Wong, *Trends Pharmacol. Sci.*, 2006, **27**, 297–309.
2 J. Folkman, *Nat. Med.*, 1995, **1**, 27–31.
3 H. K. Avraham, T. H. Lee, Y. Koh, T. A. Kim, S. Jiang, M. Sussman, A. M. Samarel and S. Avraham, *J. Biol. Chem.*, 2003, **278**, 36661–36668.
4 J. Jeon, J. Lee, C. Kim, Y. An and C. Choi, *Microvasc. Res.*, 2010, **80**, 303–309.
5 T. K. Maiti, J. Chatterjee and S. Dasgupta, *Biochem. Biophys. Res. Commun.*, 2003, **308**, 64–67.
6 L. A. Liotta, P. S. Steeg and W. G. Stetler-Stevenson, *Cell*, 1991, **64**, 327–336.
7 J. A. Forsythe, B. H. Jiang, N. V. Iyer, F. Agani, S. W. Leung, R. D. Koos and G. L. Semenza, *Mol. Cell. Biol.*, 1996, **16**, 4604–4613.
8 Y. Huang, X. Chen, K. M. Dikov, S. V. Novitskiy, C. A. Mosse, L. Yang and D. P. Carbone, *Blood*, 2007, **110**, 624–631.
9 J. F. Morère, J. M. Brechot and R. Etessami, *Targeted Oncol.*, 2006, **1**, 215–219.
10 C.-M. Lin, H. Chang, Y.-H. Chen, S. Y. Li, I.-H. Wu and J.-H. Chiu, *Int. Immunopharmacol.*, 2006, **6**, 1690–1698.
11 C. Kandaswami, L. T. Lee, P. P. Lee, J. J. Hwang, F. C. Ke, Y. T. Huang and M. T. Lee, *in Vivo*, 2005, **19**, 895–909.
12 M. Osada, S. Imaoka and Y. Funae, *FEBS Lett.*, 2004, **575**, 59–63.
13 T. Fotsis, M. Pepper, H. Adlercreutz, G. Fleischamann, T. Hase, R. Montesano and L. Scheweigerer, *Proc. Natl. Acad. Sci. U. S. A.*, 1993, **90**, 2690–2694.
14 R. Guimarães, L. Barros, M. Dueñas, R. C. Calhelha, A. M. Carvalho, C. Santos-Buelga, M. J. R. P. Queiroz and I. C. F. R. Ferreira, *Food Chem.*, 2013, **136**, 718–725.
15 P. Soares, R. Costa, H. J. C. Froufe, R. C. Calhelha, D. Peixoto, I. C. F. R. Ferreira, R. M. V. Abreu, R. Soares and M. J. R. P. Queiroz, *BioMed Res. Int.*, 2013, 154856.
16 N. M. O'Boyle1, M. Banck and C. A. James, http://www.jcheminf.com/content/3/1/33/-ins3; C. Morley, T. Vandermeersch and G. R. Hutchison, *J. Cheminf.*, 2011, **3**, 33.
17 M. F. Sanner, *Structure*, 2005, **13**, 447–462.
18 O. Trott and A. J. Olson, *J. Comput. Chem.*, 2010, **31**, 455–461.
19 R. M. Abreu, H. J. Froufe, M. J. R. P. Queiroz and I. C. F. R. Ferreira, *J Cheminf.*, 2010, **2**, 10.
20 P. Mukherjee, F. Shah, P. Desai and M. Avery, *J. Chem. Inf. Model.*, 2011, **51**, 1376–1392.
21 D. Stagos, G. D. Amoutzias, A. Matakos, A. Spyrou, A. M. Tsatsakis and D. Kouretas, *Food Chem. Toxicol.*, 2012, **50**, 2155–2170.
22 X. Cai, T. Ye, C. Liu, W. Lu, M. Lu, J. Zhang, M. Wang and P. Cao, *Toxicol. in Vitro*, 2011, **25**, 1385–1391.

7.3 “Infusion and decoction of wild German chamomile: bioactivity and characterization of organic acids and phenolic compounds”

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Infusion and decoction of wild German chamomile: Bioactivity and characterization of organic acids and phenolic compounds

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ABSTRACT

Natural products represent a rich source of biologically active compounds and are an example of molecular diversity, with recognised potential in drug discovery. Herein, the methanol extract of *Matricaria recutita* L. (German chamomile) and its decoction and infusion (the most consumed preparations of this herb) were submitted to an analysis of phytochemicals and bioactivity evaluation. The antioxidant activity was determined by free radicals scavenging activity, reducing power and inhibition of lipid peroxidation; the antitumour potential was tested in human tumour cell lines (breast, lung, colon, cervical and hepatocellular carcinomas), and the hepatotoxicity was evaluated using a porcine liver primary cell culture (non-tumour cells). All the samples revealed antioxidant properties. The decoction exhibited no antitumour activity (GI_{50} > 400 μg/mL) which could indicate that this bioactivity might be related to compounds (including phenolic compounds) that were not extracted or that were affected by the decoction procedure. Both plant methanol extract and infusion showed inhibitory activity to the growth of HCT-15 (GI_{50} 250.24 and 298.23 μg/mL, respectively) and HeLa (GI_{50} 259.36 and 277.67 μg/mL, respectively) cell lines, without hepatotoxicity (GI_{50} > 400 μg/mL). Infusion and decoction gave higher contents of organic acids (24.42 and 23.35 g/100 g dw). Otherwise, the plant methanol extract contained the highest amounts of both phenolic acids (3.99 g/100 g dw) and flavonoids (2.59 g/100 g dw). The major compound found in all the preparations was luteolin *O*-acylhexoside. Overall, German chamomile contains important phytochemicals with bioactive properties (mainly antitumour potential selective to colon and cervical carcinoma cell lines) to be explored in the pharmaceutical, food and cosmetics industries.

1. Introduction

German chamomile (*Matricaria recutita* L.) is an annual herbaceous flowering plant native to Europe. This herb has been used as a herbal remedy for thousands of years (Crevin & Philpott, 1990). It has been used traditionally as a medicinal and pharmaceutical preparation, due to its anti-inflammatory, anti-spasmodic, analgesic, antibacterial, anti-allergic antioxidant and mild astringent properties, and as a healing medicine (Maschi, Dal Cero, Galli, & Dell' Agli, 2008; McKay & Blumberg, 2006; Weiss, 1988). Externally, chamomile has been used to treat diaper rash, cracked nipples, chicken pox, ear and eye infections, disorders of the eyes including blocked tear ducts, conjunctivitis, nasal inflammation and poison ivy (Srivastava, Shankar, & Gupta, 2010).

The use of German chamomile teas as medicinal preparations has a long tradition in a variety of countries. Infusions and essential oils are used in a number of commercial products including soaps, detergents, perfumes, lotions, ointments, hair products, baked goods, confections, alcoholic beverages and herbal teas (Gupta, Mittal, Bansal, Khokra, & Kaushik, 2010). Traditionally, chamomile flowers are prepared as an infusion with water to make an herbal tea (Harbourne, Jacquier, & O'Riordan, 2009). Recent research supports this use and shows that these properties are partly due to the phenolic content (Maschi et al., 2008; McKay & Blumberg, 2006).

German chamomile contains several classes of biologically active compounds including essential oils (Granzera, Schneider, & Stuppner, 2006; Petronilho, Maraschin, Coimbra, & Rocha, 2012) and several polyphenols (McKay & Blumberg, 2006; Nováková, Vildová, Mateus, Gonçalves, & Solich, 2010). Some phenolic compounds have the capacity to quench lipid peroxidation products, prevent DNA oxidative damage, and scavenge reactive oxygen species (ROS) such as superoxide, hydrogen peroxide and hydroxyl radicals (Kahkonen et al., 1999). Flavonoids are the most abundant phenolic compounds in herbs (Mladěnka, Zatloukalová, Filipský, & Hrdina, 2010) and their effects on human health are very often

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ascribed to their potential ability to act by diminishing free radical steady-state concentration in biological systems and so providing antioxidant protection (Galleano, Verstraeten, Oteiza, & Fraga, 2010). Such ability could be possible considering that polyphenols have chemical structures supporting the scavenging of free radicals and the chelation of redox-active metals. In parallel, it has been reported that certain flavonoids can provide benefits in pathological situations associated with high free radical production, (e.g. hypertension and cardiovascular disease) (Galleano et al., 2010).

Some related studies dealing with *M. recutita* flowers are available in the literature, including reports on antioxidant properties of its methanol extract (Barros, Oliveira, Carvalho, & Ferreira, 2010; Miliauskas, Venskutonis, & van Beek, 2004), antitumour potential of aqueous and organic extracts (Srivastava & Gupta, 2007, 2009), and phenolic composition of methanolic extracts (Mulinacci, Romani, Pinelli, Vincieri, & Prucher, 2000; Nováková et al., 2010). Nevertheless, studies on *M. recutita* infusion and decoction, the most consumed preparation of this herb, are scarce, and, therefore, the present study reports the bioactive properties (antioxidant and antitumour activities, and hepatotoxicity), organic acids and phenolic characterisation of wild *M. recutita* infusions and decoction.

2. Materials and methods

2.1. Standards and reagents

Acetonitrile 99.9% was of HPLC grade from Fisher Scientific (Lisbon, Portugal). The phenolic compound standards (chlorogenic acid; ferulic acid, luteolin-6-*C*-glucoside; luteolin-7-*O*-glucoside; myricetin; protocatechuic acid and quercetin 3-*O*-glucoside) were from Extrasynthese (Genay, France). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Foetal bovine serum (FBS), L-glutamine, Hank's balanced salt solution (HBSS), trypsin–EDTA (ethylenediaminetetraacetic acid), nonessential amino acids solution (2 mM), penicillin/streptomycin solution (100 U/mL and 100 mg/mL, respectively), RPMI-1640 and DMEM media were from Hyclone (Logan, USA). Acetic acid, ellipticine, sulforhodamine B (SRB), trypan blue, trichloroacetic acid (TCA), Tris and all organic acids standards (L-ascorbic acid; citric acid, fumaric acid, malic acid, shikimic acid; succinic acid; oxalic acid and quinic acid) were from Sigma Chemical Co. (Saint Louis, USA). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

2.2. Sample

Matricaria recutita flower heads and leafy flowering stems of about 15 cm long were collected in 2009, in late spring and early summer, in the Natural Park of Montesinho territory, Trás-os-Montes, North-eastern Portugal. Key morphological characters from Rothmaler (2007) were used for plant identification. Voucher specimens are deposited in the Escola Superior Agrária de Bragança herbarium (BRESA). The sample was lyophilized (FreeZone 4.5, Labconco, Kansas, USA), reduced to a fine dried powder (20 mesh) and mixed to obtain an homogenous sample.

2.3. Infusion, decoction and methanol extract preparation

To prepare the infusion, the sample (1 g of lyophilized flowers and leafy flowering stems) was added to 200 mL of boiling distilled water and left to stand at room temperature for 5 min, and then filtered under reduced pressure. The infusion obtained was frozen and lyophilized.

To prepare the decoction, the sample (1 g) was added to 200 mL of distilled water, heated (heating plate, VELP scientific) and boiled for 5 min. The mixture was left to stand at room temperature for 5 min more, and then filtered under reduced pressure. The decoction obtained was frozen and lyophilized.

A methanol extract was also obtained from the lyophilized plant material and used as control. The sample (1 g) was extracted by stirring with 25 mL of methanol (25 °C at 150 rpm) for 1 h and subsequently filtered through Whatman No. 4 paper. The residue was then extracted with 25 mL of methanol (25 °C at 150 rpm) for 1 h. The combined methanolic extracts were evaporated at 40 °C (rotary evaporator Büchi R-210) to dryness.

2.4. Evaluation of bioactivity

2.4.1. Antioxidant activity

The lyophilized infusion and decoction and the plant methanol extract were redissolved in water (final concentration 2.5 mg/mL); the final solution was further diluted to different concentrations to be submitted for antioxidant activity evaluation by *in vitro* assays. DPPH radical-scavenging activity was evaluated using a ELX800 microplate Reader (Bio-Tek Instruments, Inc., Winooski, USA), and calculated as a percentage of DPPH discolouration using the formula: $[(A_{DPPH} - A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution containing the sample at 515 nm, and A_{DPPH} is the absorbance of the DPPH solution (Pereira, Barros, Martins, & Ferreira, 2012). Reducing power was evaluated by the capacity to convert Fe^{3+} into Fe^{2+}, measuring the absorbance at 690 nm in the microplate Reader mentioned above (Pereira et al., 2012). Inhibition of β-carotene bleaching was evaluated by the β-carotene/linoleate assay; the neutralization of linoleate free radicals avoids β -carotene bleaching, which is measured by the formula: β-carotene absorbance after 2 h of assay/initial absorbance) × 100 (Pereira et al., 2012). Lipid peroxidation inhibition in porcine (*Sus scrofa*) brain homogenates was evaluated by measuring the decrease in thiobarbituric acid reactive substances (TBARS); the colour intensity of the malondialdehyde–thiobarbituric acid (MDA–TBA) was measured by its absorbance at 532 nm; the inhibition ratio (%) was calculated using the following formula: $[(A - B)/A] \times 100\%$, where A and B were the absorbance of the control and the sample solution, respectively (Pereira et al., 2012). The results were expressed in EC_{50} value (sample concentration providing 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay). Trolox was used as a positive control.

2.4.2. Antitumour activity

The lyophilized infusion and decoction, and the plant methanol extract were redissolved in water (final concentration 8 mg/mL); the final solution was further diluted to different concentrations to be submitted to *in vitro* antitumour activity evaluation. Five human tumour cell lines were used: MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer), HCT-15 (colon carcinoma), HeLa (cervical carcinoma) and HepG2 (hepatocellular carcinoma). Cells were routinely maintained as adherent cell cultures in RPMI-1640 medium containing 10% heat-inactivated FBS (MCF-7, NCI-H460 and HCT-15) and 2 mM glutamine or in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin (HeLa and HepG2 cells), at 37 °C, in a humidified air incubator containing 5% CO_2. Each cell line was plated at an appropriate density (7.5×10^3 cells/well for MCF-7, NCI-H460 and HCT-15 or 1.0×10^4 cells/well for HeLa and HepG2) in 96-well plates and allowed to attach for 24 h. Cells were then treated for 48 h with the different diluted sample solutions. Following this incubation period, the adherent cells were fixed by adding cold 10% trichloroacetic acid (TCA, 100 μL) and incubated for 60 min at 4 °C. Plates were then washed with deionized water and dried; sulforhodamine B solution (0.1% in 1% acetic acid, 100 μL) was then added to each plate well and incubated for 30 min at room

temperature. Unbound SRB was removed by washing with 1% acetic acid. Plates were air dried, the bound SRB was solubilised with 10 mM Tris (200 μL, pH 7.4) and the absorbance was measured at 540 nm (Monks et al., 1991) in the microplate reader mentioned above. The results were expressed in GI_{50} values (sample concentration that inhibited 50% of the net cell growth). Ellipticine was used as positive control.

2.4.3. Hepatotoxicity

A cell culture was prepared from a freshly harvested porcine liver obtained from a local slaughter house. It was designed as PLP2. Briefly, the liver tissues were rinsed in Hank's balanced salt solution containing 100 U/mL penicillin and 100 μg/mL streptomycin and divided into 1×1 mm^3 explants. Some of these explants were placed in 25 cm^2 tissue flasks in DMEM supplemented with 10% fetal bovine serum, 2 mM nonessential amino acids and 100 U/mL penicillin, 100 mg/mL streptomycin and incubated at 37 °C with a humidified atmosphere containing 5% CO_2. The medium was changed every 2 days. Cultivation of the cells was continued with direct monitoring every 2–3 days using a phase contrast microscope. Before confluence, cells were subcultured and plated in 96-well plates at a density of 1.0×10^4 cells/well, and cultivated in DMEM medium with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin (Abreu et al., 2011). Cells were treated for 48 h with the different diluted sample solutions and the same procedure described in the previous section for SRB assay was followed. The results were expressed in GI_{50} values (sample concentration that inhibited 50% of the net cell growth). Ellipticine was used as positive control.

2.5. Analysis of organic acids

Organic acids were determined following a procedure previously optimized and described by the authors (Barros, Pereira, & Ferreira, in press). Analysis was performed by ultra fast liquid chromatography (UFLC) coupled to photodiode array detector (PDA), using a Shimadzu 20A series UFLC (Shimadzu Cooperation). Detection was carried out in a PDA, using 215 and 245 nm as preferred wavelengths. The organic acids were quantified by comparison of the area of their peaks recorded at 215 nm with calibration curves obtained from commercial standards of each compound. The results were expressed in g per 100 g of dry weight (dw).

2.6. Analysis of phenolic compounds

Phenolic compounds were determined by HPLC (Hewlett-Packard 1100, Agilent Technologies, Santa Clara, USA) as previously described by the authors (Barros et al., 2012). Double online detection was carried out in the diode array detector (DAD) using 280 and 370 nm as preferred wavelengths and in a mass spectrometer (MS) connected to the HPLC system via the DAD cell outlet. The phenolic compounds were characterized according to their UV and mass spectra and retention times, and by comparison with authentic standards when available. For quantitative analysis, calibration curves were prepared from different standard compounds. The results were expressed in g per 100 g of dry weight (dw).

2.7. Statistical analysis

All the assays were carried out in triplicate in three different samples of infusion, decoction and plant methanol extract; the results of bioactivity (antioxidant and antitumour activities, and hepatotoxicity) and of organic acids and phenolic compounds composition are expressed as mean values ± standard deviation (SD). The statistical differences represented by letters were obtained through one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference post hoc test with $\alpha = 0.05$. These treatments were carried out using SPSS v. 18.0 programme.

3. Results and discussion

3.1. Evaluation of bioactivity

The antioxidant activity was determined by free radicals scavenging activity, reducing power and inhibition of lipid peroxidation in brain cell homogenates. The antitumour potential was tested in human tumour cell lines (breast, lung, colon, cervical and hepatocellular carcinomas) and the hepatotoxicity was evaluated using a porcine liver primary cell culture. The results are shown in Table 1.

The infusion and decoction lyophilized samples gave similar antioxidant activity properties and were better than the plant methanol extract in the radical scavenging assays (DPPH and β-carotene bleaching inhibition). Otherwise, the plant methanol extract gave higher reducing power and lipid peroxidation inhibition measured by TBARS assay (lowest EC_{50} values). The mechanisms involved in the assays used to evaluate antioxidant activity are different and, therefore, each plant preparation can have different compounds with specific capacities to participate in those mechanisms.

Trolox and ellipticine were used as positive controls of antioxidant and antitumour activities evaluation assays, but should not be considered as standards and the comparison with extracts/oral preparations results should be avoided, because they are individual compounds and not mixtures.

Regarding antitumour effects, *M. recutita* infusion and plant methanol extract was selective for HCT-15 and HeLa, since no activity was observed against the other cell lines: MCF-7, NCI-H460 and HepG2. Nevertheless, none of the *M. recutita* preparations showed hepatotoxicity in the porcine liver primary cell culture (non-tumour cells; PLP2) (Table 1). The plant methanol extract was slightly more potent than the infusion sample in HCT-15 and HeLa human cell lines. The decoction preparation had no antitumour effects at the maximal concentration used (400 μg/mL). The results obtained are in agreement with other authors that reported minimal growth inhibitory effects in normal cells, but a significant reduction in cell viability in various human cancer cell lines, mainly from methanolic fractions rather than aqueous fractions (Srivastava & Gupta, 2007; Srivastava & Gupta, 2009). The absence of antitumour activity in the decoction of another chamomile species, *Chamaemelum nobile* (Roman chamomile), was previously reported by the authors (Guimarães et al., 2013).

It should be highlighted that *M. recutita* has been included in commercial mixtures for different pharmacological applications such as (i) TBS-101 (a mixture of seven standardized botanical extracts) that showed an outstanding safety profile with significant anticancer activity against androgen-refractory human prostate cancer PC-3 cells, both *in vitro* and *in vivo* (Evans, Dizeyi, Abrahamsson, & Persson, 2009); (ii) STW 5 (a mixture of nine standardized botanical extracts) for treatment of gastrointestinal disorders, with a mechanism of action related to their antioxidant properties (Schempp, Weiser, Kelber, & Elstner, 2006). Nevertheless, chemical characterization and bioactivity evaluation of *M. recutita* infusion and decoction, the most consumed preparations of this herb, have been discarded being addressed herein.

3.2. Analysis of organic acids and phenolic compounds

Oxalic, quinic, malic, citric and succinic acids were quantified in all the extracts of *M. recutita*, malic acid being the most abundant organic acid (Table 2). Shikimic and fumaric acids were present in

Table 1
Antioxidant and antitumour activities, and hepatotoxicity of wild *Matricaria recutita* (mean ± SD).

	Infusion	Decoction	Plant methanol extract*	Positive control**
Extraction yield (%)	16.25 ± 0.59	19.37 ± 1.09	16.09 ± 0.77	–
Antioxidant activity (EC_{50} values, μg/mL)				
DPPH scavenging activity	394.97 ± 44.31^b	344.02 ± 18.65^b	800.36 ± 49.09^a	43.03 ± 1.71
Reducing power	316.61 ± 2.46^a	318.75 ± 3.01^a	232.49 ± 26.19^b	29.62 ± 3.15
β-Carotene bleaching inhibition	422.72 ± 92.91^b	497.34 ± 107.67^b	661.11 ± 21.93^a	2.63 ± 0.14
TBARS inhibition	511.01 ± 17.28^a	508.44 ± 4.43^a	183.48 ± 3.52^b	3.73 ± 1.90
Antitumour activity (GI_{50} values, μg/mL)				
MCF-7 (breast carcinoma)	>400	>400	>400	0.91 ± 0.04
NCI-H460 (non-small lung cancer)	>400	>400	>400	1.42 ± 0.00
HCT-15 (colon carcinoma)	298.23 ± 11.58^a	>400	250.24 ± 18.38^b	1.91 ± 0.06
HeLa (cervical carcinoma)	277.67 ± 9.04^a	>400	259.36 ± 7.57^b	1.14 ± 0.21
HepG2 (hepatocellular carcinoma)	>400	>400	>400	3.22 ± 0.67
Hepatotoxicity (GI_{50} value, μg/mL)				
PLP2	>400	>400	>400	2.06 ± 0.03

EC_{50} values correspond to the sample concentration achieving 50% of antioxidant activity or 0.5 of absorbance in reducing power assay.
GI_{50} values correspond to the sample concentration achieving 50% of growth inhibition in human tumour cell lines or in liver primary culture PLP2.
In each row different letters mean significant differences ($p < 0.05$).
* Results reported in Barros et al. (2012).
** Trolox and ellipticine for antioxidant and antitumour activity assays, respectively.

low or vestigial amounts. Ascorbic acid was not detected, neither in the infusion nor in the decoction, and was present in traces in the plant extract. Both the infusion and decoction contained similar contents of organic acids, which, in both cases, were higher than in the plant extract.

Organic acids might have antioxidant properties, as is the case with citric and oxalic acids (Hraš, Halodin, Knez, & Bauman, 2000; Kayashima & Katayama, 2002), which may contribute to the antioxidant activity of the samples studied herein.

The main phenolic compounds found in *M. recutita* plant and in its decoction and infusion were phenolic acids and derivatives, and also flavonoids such as flavonols and flavones (Tables 3 and 4). The chromatographic profile of the three plant preparations can be seen in Fig. 1. Up to 20 phenolic compounds, including phenolic acids and flavonoids, were detected in the *M. recutita* preparations (Table 3).

Compound **2** was identified as protocatechuic acid by comparing its UV spectrum and retention time with those of a commercial standard. Thirteen hydroxycinnamic acid derivatives (peaks 1, 3–9, 11, 12, 14, 16 and 19) were detected, 10 being quinic acid derivatives (1, 3–6, 9, 12, 14, 16 and 19), whose identities were assigned based on their MS spectra and fragmentation patterns. These compounds released characteristic MS^2 fragment ions at *m/z* 191 (deprotonated quinic acid), 179 (deprotonated caffeic acid) or 193 (deprotonated ferulic acid), which, together with their pseudo molecular ions $[M-H]^-$ at *m/z* 353, 515, 677 or 367, allowed their identification as quinic acid derivatives containing one, two or three caffeic acid moieties or one ferulic acid moiety, respectively.

Table 2
Identification and quantification of organic acids in wild *Matricaria recutita* (mean ± SD).

Organic acid	Infusion	Decoction	Plant (control)
Oxalic acid	8.45 ± 0.32^a	8.60 ± 0.47^a	3.24 ± 0.05^b
Quinic acid	0.24 ± 0.00^b	0.88 ± 0.19^a	0.17 ± 0.00^c
Malic acid	2.26 ± 0.06^a	1.97 ± 0.03^b	0.39 ± 0.02^c
Shikimic acid	0.02 ± 0.00	0.02 ± 0.00	Tr
Ascorbic acid	Nd	Nd	Tr
Citric acid	6.44 ± 0.85^a	6.14 ± 0.14^a	1.55 ± 0.00^b
Succinic acid	7.00 ± 0.21^a	5.74 ± 0.13^b	1.94 ± 0.05^c
Fumaric acid	0.01 ± 0.00	0.01 ± 0.00	Tr
Total (g/100 g dw)	24.42 ± 1.32^a	23.35 ± 0.65^a	7.30 ± 0.03^b

In each row different letters mean significant differences ($p < 0.05$). Tr, traces; nd, not detected; dw, dry weight.

The assignments of the different caffeoylquinic acid isomers were made using the recommended IUPAC numbering system and the hierarchical keys previously developed by Clifford, Johnston, Knight, and Kuhnert (2003) and Clifford, Knight, and Kuhnert (2005).

Compound **6** was positively identified as 5-*O*-caffeoylquinic acid by comparison with an authentic standard, and also due to its MS fragmentation pattern. Compound **5** was tentatively assigned as the corresponding *cis* isomer of 5-*O*-caffeoylquinic acid based on its fragmentation pattern and lower levels compared with peak 6. Furthermore, hydroxycinnamoyl *cis* derivatives would be expected to elute before the corresponding *trans* derivatives, as observed after UV irradiation (366 nm, 24 h) of hydroxycinnamic acids in our laboratory.

Compound **1** ($[M-H]^-$ at *m/z* 353) was identified as 3-*O*-caffeoylquinic acid, yielding the base peak at *m/z* 191 and the ion at *m/z* 179 with an intensity >55% of the base peak, characteristic of 3-acylchlorogenic acids as reported by Clifford et al. (2003), Clifford et al. (2005). Compound **4** was easily distinguished from the other two isomers by its base peak at *m/z* 173 [quinic acid–H–H_2O]$^-$, accompanied by a secondary fragment ion at *m/z* 179 with approximately 84% of the abundance of the base peak, which allowed its identification as 4-*O*-caffeoylquinic acid according to the fragmentation pattern described by Clifford et al. (2003), Clifford et al. (2005). Compound **9** was tentatively identified as 5-*O*-feruloylquinic acid taking into account its fragmentation pattern and relative intensities similar to those of 5-*O*-caffeoylquinic acid.

Compounds **3**, **14**, 16 and **19** ($[M-H]^-$ at *m/z* 515) could be assigned as dicaffeoylquinic acids and were assigned as 1,5-*O*-, 3,4-*O*-, 3,5-*O*- and 4,5-*O*-dicaffeoylquinic acids, respectively, based on their elution order, fragmentation pattern and relative abundances (Clifford et al., 2003; Clifford et al., 2005). MS^2 fragmentation of compound **14** yielded the formation of signals corresponding to "dehydrated" fragment ions at *m/z* 335 [caffeoylquinic acid–H–H_2O]$^-$ and *m/z* 173 [quinic acid–H–H_2O]$^-$, characteristic of 4-acyl-caffeoylquinic acids. Furthermore, according to Clifford et al. (2005), the intensity of signal at *m/z* 335 (25% of base peak) is more intense than in 4,5-*O*-dicaffeoylquinic (barely detectable, 4% of base peak). These observations permitted the assignment of compound 14 as 3,4-*O*-dicaffeoylquinic acid. The fragmentation pattern for 3,5-*O*-dicaffeoylquinic (compound **16**) acid was similar to the one previously reported by Clifford et al. (2005). MS^2 base peak was at *m/z* 353, produced by the loss of one of the caffeoyl moieties [M–H–caffeoyl]$^-$, and subsequent fragmentation of this

Table 3
Retention time (Rt), wavelengths of maximum absorption in the UV–vis region (λ_{max}), pseudomolecular and MS^2 fragment ions (in brackets, relative abundances) and tentative identification of the phenolic compounds of wild *Matricaria recutita*.

Peak	Rt (min)	λ_{max} (nm)	Molecular ion [M-H]$^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification
1	7.69	328	353	191(100), 179(53), 173(5), 161(5), 135(48)	3-*O*-caffeolyquinic acid
2	8.24	294	153	109(100)	Protocatechuic acid
3	9.75	320	515	515(100), 353(5), 191(33), 179(6), 161(13)	1,5-Dicaffeolyquinic acid
4	10.84	326	353	191(75), 179(84), 173(100), 161(11), 135(77)	4-*O*-Caffeolyquinic acid
5	11.34	326	353	191(100), 179(7), 173(3), 161(4), 135(2)	*cis*-5-*O*-Caffeolyquinic acid
6	11.57	326	353	191(100), 179(15), 173(8), 161(15), 135(7)	*trans*-5-*O*-Caffeolyquinic acid
7	12.24	312	355	193(100), 179(13), 149(80)	*cis*-Ferulic acid hexoside
8	12.39	312	355	193(100), 179(13), 149(81)	*trans*-Ferulic acid hexoside
9	14.85	328	367	193(16), 191(100), 173(19), 134(8)	5-*O*-Feruloylquinic acid
10	15.18	354	479	317(100)	Myricetin *O*-hexoside
11	15.45	322	711	549(3), 355(36), 193(100), 149(84)	Ferulic acid hexoside dimer
12	15.81	322	677	515(100), 497(2), 353(17), 335(7), 191(4), 179 (7), 173 (1), 135 (3)	1,3,5-*O* or 1,4,5-*O*-Tricaffeolyquinic
13	16.54	340	651	489(96), 447(55), 285(45)	Luteolin acetylhexoside hexoside
14	17.11	330	515	515 (100), 353(66), 335(25), 299(2), 255(3), 203(3), 191(26), 179(53), 173(68), 161(9)	3,4-*O*-Dicaffeolyquinic acid
15	17.68	340	447	285(100)	Luteolin-7-*O*-glucoside
16	18.09	330	515	353(100), 335(8), 191(89), 179(75), 173(11), 161(7), 155(2), 135(28)	3,5-*O*-Dicaffeolyquinic acid
17	18.54	372	505	343(8), 301(100)	Quercetin 7-*O*-acetylhexoside
18	18.64	343	489	327(5), 285(64)	Luteolin *O*-acylhexoside
19	18.81	328	515	353(100), 335(4), 299(5), 255(5), 203(3), 191(30), 179(67), 173(95), 161(2), 155(3), 135(24)	4,5-*O*-Dicaffeoylquinic acid
20	19.10	296(sh), 336	489	327(5), 285(64)	Luteolin *O*-acylhexoside

Table 4
Quantification of phenolic compounds in wild *Matricaria recutita* (mean ± SD).

Peak	Infusion	Decoction	Plant (control)
3-*O*-caffeolyquinic acid	0.15 ± 0.01^a	0.12 ± 0.00^b	0.07 ± 0.00^c
Protocatechuic acid	0.07 ± 0.01^a	0.05 ± 0.01^b	Nd
1,5-Dicaffeolyquinic acid	Nd	Tr	0.02 ± 0.00
4-*O*-Caffeolyquinic acid	0.21 ± 0.00^b	0.13 ± 0.00^c	0.24 ± 0.00^a
cis-5-*O*-Caffeolyquinic acid	0.17 ± 0.04^a	0.15 ± 0.00^a	Nd
trans-5-*O*-Caffeolyquinic acid	0.26 ± 0.04^b	0.22 ± 0.01^b	1.02 ± 0.02^a
cis-Feruloyl hexoside acid	0.32 ± 0.02^a	0.28 ± 0.01^b	Nd
trans-Feruloyl hexoside acid	0.46 ± 0.01^b	0.38 ± 0.01^c	1.02 ± 0.00^a
5-*O*-Feruloylquinic acid	0.03 ± 0.00^a	0.02 ± 0.00^b	Nd
Myricetin *O*-hexoside	Nd	Nd	0.05 ± 0.00
Feruloyl hexoside acid dimer	0.59 ± 0.00^b	0.55 ± 0.01^c	0.91 ± 0.00^a
1,3,5-*O* or 1,4,5-Tricaffeolyquinic	0.03 ± 0.00^b	0.02 ± 0.00^c	0.06 ± 0.00^a
Luteolin acetylhexoside hexoside	0.02 ± 0.00^c	0.03 ± 0.00^b	0.11 ± 0.01^a
3,4-*O*-Dicaffeolyquinic acid	0.73 ± 0.03^a	0.33 ± 0.01^b	0.35 ± 0.00^b
Luteolin-7-*O*-glucoside	0.17 ± 0.01^a	0.09 ± 0.00^b	0.06 ± 0.00^c
3,5-*O*-Dicaffeolyquinic acid	0.26 ± 0.07^a	0.16 ± 0.00^b	0.10 ± 0.00^b
Quercetin 7-*O*-acetylhexoside	Nd	Nd	0.10 ± 0.00
Luteolin *O*-acylhexoside	0.09 ± 0.01^b	0.06 ± 0.00^c	0.19 ± 0.01^a
4,5-*O*-Dicaffeoylquinic acid	0.17 ± 0.03^a	0.13 ± 0.01^b	Nd
Luteolin *O*-acylhexoside	1.29 ± 0.12^b	0.81 ± 0.04^c	2.10 ± 0.00^a
Total phenolic acids (g/100 g dw)	3.43 ± 0.22^b	2.53 ± 0.02^c	3.99 ± 0.02^a
Total flavonoids (g/100 g dw)	1.56 ± 0.12^b	0.98 ± 0.04^c	2.59 ± 0.01^a
Total phenolic compounds (g/100 g dw)	5.00 ± 0.33^b	3.51 ± 0.06^c	6.58 ± 0.03^a

In each row different letters mean significant differences ($p < 0.05$). Tr, traces; nd, not detected; dw, dry weight.

ion yielded the same fragments as 5-caffeoylquinic acid at *m/z* 191, 179 and 135, although in this case with a comparatively more intense signal at *m/z* 179 [caffeic acid–H]$^-$ (75% base peak). Compound 19 was assigned as 4,5-*O*-dicaffeoylquinic acid since its fragmentation was identical to those previously reported by Clifford et al. (2005). In this case, the signal at *m/z* 335 was barely detectable (<5% of base peak) and the intense signal at *m/z* 173 is characteristic of an isomer substituted at position 4. This indicated that whereas 3,4-*O*-dicaffeoylquinic acid initially loses the caffeoyl moiety at position 3, 4,5-*O*-dicaffeoylquinic acid would initially lose that moiety at position 5. Compound **3** was assigned as 1,5-*O*-dicaffeoylquinic acid, following the criteria reported by Clifford et al. (2005), including the weak ions at *m/z* 335 and 179 (<10% of base peak).

Compound **12** was identified as tricaffeoylquinic acid according to its pseudomolecular ion [M–H]$^-$ at *m/z* 677 and diagnostic MS^2 fragments at *m/z* 515 (loss of the first caffeoyl), *m/z* 353 (loss of the second caffeoyl), *m/z* 191 (loss of the third caffeoyl to give quinic acid ion), *m/z* 179 (caffeic acid ion). The signal observed at *m/z* 497 can be interpreted as being due to the loss of a water molecule from the ion at *m/z* 515. According to the relative intensities of different tricaffeoylquinic acid isomers reported by Lin and Harnly (2008), this compound could be assigned as 1,3,5-*O*-tricaffeoylquinic acid or 1,4,5-*O*-tricaffeoylquinic acid.

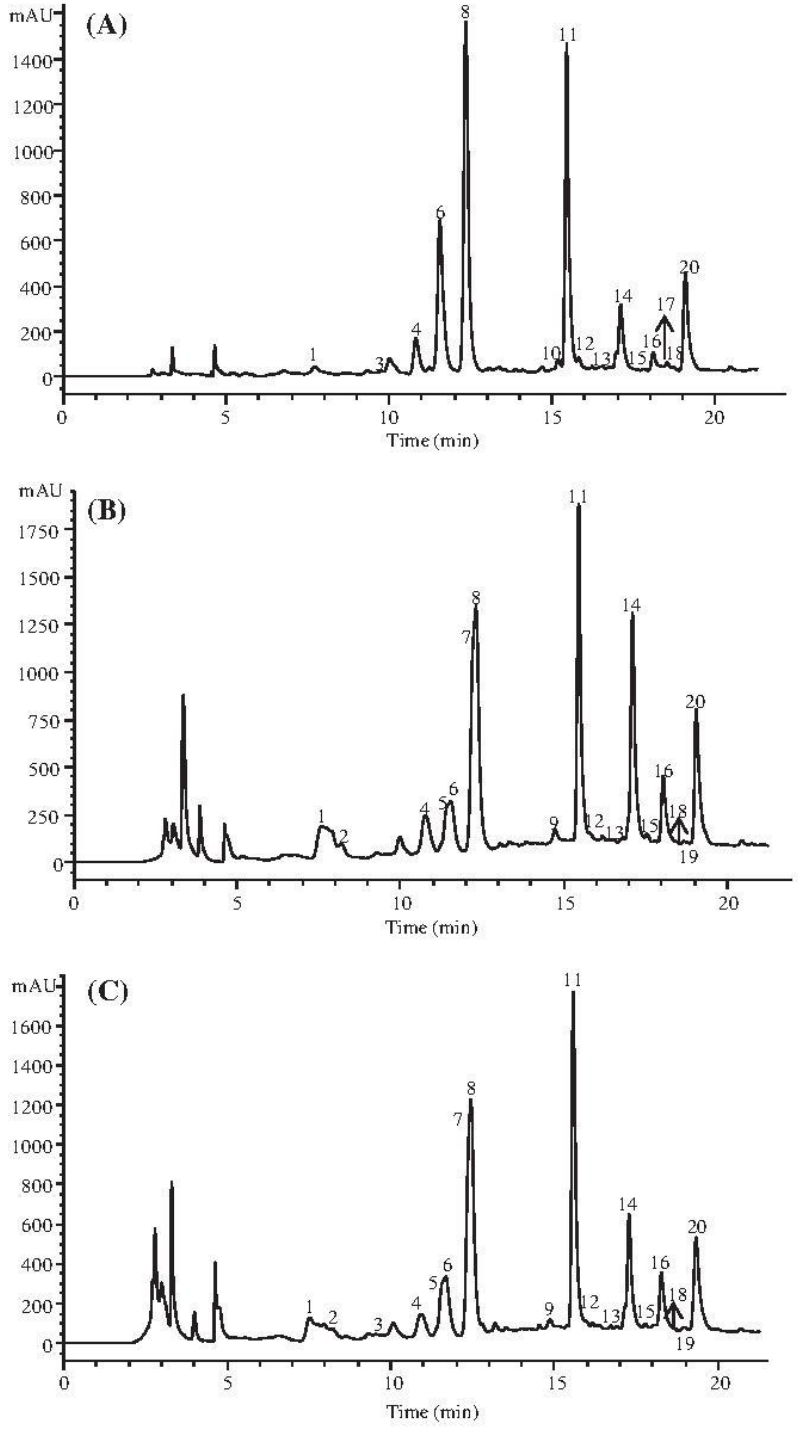

Fig. 1. HPLC chromatogram of the phenolic compounds of *Matricaria recutita* recorded at 280 nm (A) plant methanol extract (control; 1:2 *v*/*v*); (B) infusion and (C) decoction.

Compounds **7** and **8** with MS^2 fragments at *m*/*z* 193 ([ferulic acid−H]$^-$) resulting from the loss of a hexosyl moiety, -162 mu, and 176 ([ferulic acid−H−H_2O]$^-$) was tentatively assigned as a ferulic acid hexoside. Compound **7** presented similar UV and mass spectra characteristics as peak 8, but with an earlier retention time. Taking into account the observation above it was tentatively identified as *cis*-ferulic acid hexoside. Similarly, compound **11** was tentatively identified as ferulic acid hexoside dimer.

Flavones were the most abundant flavonoids present in the samples analysed (Tables 3 and 4).

Compounds **13**, **15**, 18 and **20** were identified as luteolin derivatives. Compounds **15**, 18 and **20** presented pseudomolecular ions

$[M-H]^-$ at m/z 447 and 489 releasing a common MS^2 fragment at m/z 285 ($[M-H-162]^-$ and $[M-H-42-162]^-$, loss of hexosyl and acetylhexoside moieties, respectively). They were tentatively identified as luteolin 7-*O*-glucoside (retention time compared with a commercial standard), and two luteolin *O*-acetylhexosides (compound **18** and **20**), respectively. Compound **13** presented a pseudomolecular ion $[M-H]^-$ at m/z 651 releasing three MS^2 fragments at m/z 489, 447 and 285 (loss of hexosyl, acetylhexosyl and acetyldihexosyl moieties, respectively), being tentatively identified as luteolin acetylhexoside hexoside.

Flavonols (compounds **10** and **17**) were also found in the samples (Table 3). Compound **10** presented a pseudomolecular ion $[M-H]^-$ at m/z 479, releasing an MS^2 fragment at m/z 317 ($[M-H-162]^-$, loss of an hexosyl moiety), corresponding to myricetin, which allowed a tentative identification of the compound as myricetin *O*-hexoside. Compound **17** corresponded to a quercetin derivative presenting a pseudomolecular ion $[M-H]^-$ at m/z 505 and releasing an MS^2 fragment at m/z 301 (quercetin; $[M-H-42-162]^-$, loss of an acetylhexoside moiety). It is known that the introduction of a glycoside on the hydroxyl groups at positions 7, 3′ or 4′ has no effect on wavelength maxima or the spectrum shape in relation to the aglycone (Mabry, Markham, & Thomas, 1970). Thus, quercetin 7-*O*-glycosides would have λ_{max} in Band I around 370 nm, while quercetin 3 *O*-glycosides are hypsochromically shifted to around 354 nm. Since this compound presented λ_{max} at 372 nm it was tentatively identified as quercetin 7-*O*-acetylhexoside.

The amounts of the phenolic compounds found varied among the different preparations and flavonols decreased in infusions and decoctions, but the *cis* isomer of caffeoylquinic and ferulic acids derivatives appeared in these preparations (Table 4 and Fig. 1). The plant methanol extract (control) presented the highest amounts of phenolic acids (3.99 g/100 g dw) and flavonoids (2.59 g/100 g dw) as well as total phenolic compounds (6.58 g/100 g dw), followed by the infusion (5.00 g/100 g dw) and decoction (3.51 g/100 g dw). The same was observed in *C. nobile* (Roman chamomile) in previously work from our research group (Guimarães et al., 2013).

The major compound found in the herbal plant and in the preparations was luteolin *O*-acylhexoside (compound **20**). Mulinacci et al. (2000), Nováková et al. (2010), Harbourne, Jacquier, and O'Riordan, (2009) and Srivastava and Gupta, (2009) reported the presence of apigenin 7-*O*-glucoside and other apigenin derivatives, but these compounds were not detected in our samples.

Furthermore, Mulinacci et al. (2000) revealed the presence of different flavonoids, such as patuletin and other quercetin derivatives. In relation to the quantification, no comparison can be made due to the fact that those authors only presented percent area of the main phenolic compounds found measured at 335 nm.

Nováková et al. (2010) presented the phenolic profile of methanolic extracts of *M. recutita* herbal flowers and infusions, demonstrating also lower values in an infusion preparation when compared to the herbal plant methanolic extract. These authors also found a dissimilar profile than that obtained in this study, finding other flavonoids, such as kaempferol, isorhamnetin and different quercetin derivatives in their samples. The quantification was expressed in μmol/L and this makes these results difficult to compare with the results for *M. recutita* obtained herein.

Overall, a decrease in the amount of phenolic compounds in the plant infusion or decoction was observed, compared to those in the methanol extract; the same was not observed for organic acids, which indicates that these compounds are better extracted with hot water than with methanol. The decoction had no antitumour effects which could indicate that these effects might be related to compounds (including phenolic compounds) that were not extracted or that were affected by the decoction procedure. Both plant methanol extract and infusion showed inhibitory activity of the growth of colon and cervical carcinoma cell lines, without toxicity for hepatocyte normal cells. Therefore, wild German chamomile (*M. recutita*) may be considered a source of important phytochemicals with bioactive properties to be explored in the medicine, food, and cosmetic industries.

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References

Abreu, R. M. V., Ferreira, I. C. F. R., Calhelha, R. C., Lima, R. T., Vasconcelos, M. H., Adega, F., et al. (2011). Anti-hepatocellular carcinoma activity using human HepG2 cells and hepatotoxicity of 6-substituted methyl 3-aminothieno[3,2-*b*]pyridine-2-carboxylate derivatives: In vitro evaluation, cell cycle analysis and QSAR studies. *European Journal of Medicinal Chemistry, 46*, 5800–5806.

Barros, L., Dueñas, M., Dias, M. I., Sousa, M. J., Santos-Buelga, C., & Ferreira, I. C. F. R. (2012). Phenolic profiles of *in vivo* and *in vitro* grown *Coriandrum sativum* L. *Food Chemistry, 132*, 841–848.

Barros, L., Pereira, C., & Ferreira, I.C.F.R. (in press). Optimized analysis of organic acids in edible mushrooms from Portugal by ultra fast liquid chromatography and photodiode array detection. *Food Analytical Methods*. Doi: http://dx.doi.org/10.1007/s12161-012-9443-1.

Barros, L., Oliveira, S., Carvalho, A., & Ferreira, I. C. F. R. (2010). *In vitro* antioxidant properties and characterization in nutrients and phytochemicals of six medicinal plants from the Portuguese folk medicine. *Industrial Crops and Products, 32*, 572–579.

Clifford, M. N., Johnston, K. L., Knight, S., & Kuhnert, N. A. (2003). A hierarchical scheme for LC–MS^n identification of chlorogenic acids. *Journal of Agricultural and Food Chemistry, 51*, 2900–2911.

Clifford, M. N., Knight, S., & Kuhnert, N. A. (2005). Discriminating between the six isomers of dicaffeoylquinic acid by LC–MS^n. *Journal of Agricultural and Food Chemistry, 53*, 3821–3832.

Crevin, J. K., & Philpott, J. (1990). *Herbal medicine past and present* (Vol. II). Duke University Press.

Evans, S., Dizeyi, N., Abrahamsson, P. A., & Persson, J. (2009). The effect of a novel botanical agent TBS-101 on invasive prostate cancer in animal models. *Anticancer Research, 10*, 3917–3924.

Galleano, M., Verstraeten, S. V., Oteiza, P. I., & Fraga, C. G. (2010). Antioxidant actions of flavonoids: thermodynamic and kinetic analysis. *Archives of Biochemistry and Biophysics, 501*, 23–30.

Granzera, M., Schneider, P., & Stuppner, H. (2006). Inhibitory effects of the essential oil of chamomile (*Matricaria recutita*) and its major constituents on human cytochrome P450 enzymes. *Life Sciences, 78*, 856–861.

Guimarães, R., Barros, L., Dueñas, M., Calhelha, R. C., Carvalho, A. M., Santos-Bulega, C., et al. (2013). Nutrients, phytochemicals and bioactivity of wild Roman chamomile: A comparison between the herb and its preparations. *Food Chemistry, 136*, 718–725.

Gupta, V., Mittal, P., Bansal, P., Khokra, S. L., & Kaushik, D. (2010). Pharmacological potential of *Matricaria recutita* – A review. *International Journal of Pharmaceutical Sciences and Drug research, 2*, 12–16.

Harbourne, N., Jacquier, J., & O'Riordan, D. (2009). Optimisation of the extraction and processing conditions of chamomile (*Matricaria chamomilla* L.) for incorporation into a beverage. *Food Chemistry, 115*, 15–19.

Hraš, A. R., Halodin, M., Knez, Z., & Bauman, D. (2000). Comparison of antioxidative and synergistic effects of rosemary extract with α-tocopherol, ascorbyl palmitate and citric acid in sunflower oil. *Food Chemistry, 71*, 229–233.

Kahkonen, M. P., Hopia, A. I., Vuorela, H. J., Rauha, J. P., Pihlaja, K., Kujala, T. S., et al. (1999). Antioxidant activity of plant extracts containing phenolic compounds. *Journal of Agricultural and Food Chemistry, 47*, 3954–3962.

Kayashima, T., & Katayama, T. (2002). Oxalic acid is available as a natural antioxidant in some systems. *Biochimica et Biophysica Acta, 1573*, 1–3.

Lin, L. Z., & Harnly, J. M. (2008). Identification of hydroxycinnamoylquinic acids of arnica flowers and burdock roots using a standardized LC–DAD–ESI/MS profiling method. *Journal of Agricultural and Food Chemistry, 56*, 10105–10114.

Mabry, T. J., Markham, K. R., & Thomas, M. B. (1970). *The systematic identification of flavonoids*. New York: Springer-Verlag Publication.

Maschi, O., Dal Cero, E., Galli, G. V., Caruso, D., Bosisio, E., & Dell' Agli, M. (2008). Inhibition of human cAMP-Phosphodiesterase as a mechanism of the spasmolytic effect of *Matricaria recutita* L. *Journal of Agricultural and Food Chemistry, 56*, 5015–5020.

McKay, D. L., & Blumberg, J. B. (2006). A review of the bioactivity and potential health benefits of chamomile tea (*Matricaria recutita* L.). *Phytotherapy Research, 20*, 519–530.

Miliauskas, G., Venskutonis, P. R., & van Beek, T. A. (2004). Screening of radical scavenging activity of some medicinal and aromatic plant extracts. *Food Chemistry, 85*, 231–237.

Mladěnka, P., Zatloukalová, L., Filipský, T., & Hrdina, R. (2010). Cardiovascular effects of flavonoids are not caused only by direct antioxidant activity. *Free Radical Biology & Medicine, 49*, 963–975.

Monks, A., Scudiero, D., Skehan, P., Shoemaker, R., Paull, K., Vistica, D., et al. (1991). Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. *Journal of the National Cancer Institute, 83*, 757–766.

Mulinacci, N., Romani, A., Pinelli, P., Vincieri, F. F., & Prucher, D. (2000). Characterization of *Matricaria recutita* L. flower extracts by HPLC–MS and HPLC–DAD analysis. *Chromatographia, 51*, 301–307.

Nováková, L., Vildová, A., Mateus, J. P., Gonçalves, T., & Solich, P. (2010). Development and application of UHPLC–MS/MS method for the determination of phenolic compounds in chamomile flowers and chamomile tea extracts. *Talanta, 82*, 1271–1280.

Pereira, E., Barros, L., Martins, A., & Ferreira, I. C. F. R. (2012). Towards chemical and nutritional inventory of Portuguese wild edible mushrooms in different habitats. *Food Chemistry, 130*, 394–403.

Petronilho, S., Maraschin, M., Coimbra, M. A., & Rocha, S. M. (2012). *In vitro* and *in vivo* studies of natural products: A challenge for their valuation. The case study of chamomile (*Matricaria recutita* L.). *Industrial Crops and Products, 40*, 1–12.

Rothmaler, W. (2007). *Exkursionsflora von Deutschland. Gefaesspflanzen: Atlasband* (Band 3). Munchen: Elsevier GmbH.

Schempp, H., Weiser, D., Kelber, O., & Elstner, E. F. (2006). Radical scavenging and anti-inflammatory properties of STW 5 (Iberogasts) and its components. *Phytomedicine, 13*, 36–44.

Srivastava, J. K., & Gupta, S. (2007). Antiproliferative and apoptotic effects of chamomile extract in various human cancer cells. *Journal of Agricultural and Food Chemistry, 55*, 9470–9478.

Srivastava, J. K., & Gupta, S. (2009). Extraction, characterization, stability and biological activity of flavonoids isolated from chamomile flowers. *Molecular Cell Pharmacology, 1*, 138–152.

Srivastava, J. K., Shankar, E., & Gupta, S. (2010). Chamomile: A herbal medicine of the past with a bright future (Review). *Molecular Medicine Reports, 3*, 895–901.

Weiss, R. F. (1988). Herbal medicine. In A. B. Arcanum (Ed.) (pp. 22–28). Beaconsfield: Beaconsfield Publishers.

7.4 “Caracterization of phenolic compounds in wild fruits of Northeastern Portugal”

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Analytical Methods

Characterisation of phenolic compounds in wild fruits from Northeastern Portugal

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ABSTRACT

This study aimed to analyse the phenolic composition of wild fruits of *Arbutus unedo* (strawberry-tree), *Prunus spinosa* (blackthorn), *Rosa canina* and *Rosa micrantha* (wild roses). Analyses were performed by HPLC-DAD–ESI/MS. *P. spinosa* fruits presented the highest concentration in phenolic acids (29.78 mg/100 g dry weight), being 3-*O*-caffeoylquinic acid the most abundant one, and flavone/ols (57.48 mg/100 g), among which quercetin3-*O*-rutinoside (15.63 mg/100 g) was the majority compound. (+)-Catechin was the most abundant compound in *A. unedo* (13.51 mg/100 g) and *R. canina* (3.59 mg/100 g) fruits. *A. unedo* fruits presented the highest concentration in flavan-3-ols (36.30 mg/100 g). Cyanidin 3-*O*-glucoside was found in all the studied fruits, being the major anthocyanin in most of them, with the exception of *P. spinosa* samples, in which cyaniding 3-*O*-rutinoside and peonidin 3-*O*-rutinoside predominated; *P. spinosa* fruit presented the more complex anthocyanin profile among the analysed fruits and also the highest anthocyanin concentrations, which was coherent with its greater pigmentation. All in all, *P. spinosa* presented the highest levels of phenolic acids and flavonoids, including anthocyanins, flavonols and flavones, although no flavan-3-ols could be identified in its fruits. The present study represents a contribution to the chemical characterisation of phenolic compounds from wild fruits with acknowledged antioxidant activity and traditionally used for several folk medicinal applications.

1. Introduction

Plant polyphenols are a wide group of secondary metabolites and are a common component of our diet. They are widely distributed and can range from simple molecules, such as phenolic acids, to complex molecules with numerous phenolic groups, e.g. acylated flavonoid glycosides, proanthocyanidins or oligomeric hydrolysable tannins. Polyphenols occur primarily in conjugated form, linked to sugars moieties, but also to other compounds, such as carboxylic and organic acids, amines, lipids and even to other polyphenols (Bravo, 1998).

Flavonoids represent the most common and widely distributed group of plant phenolics, and can be further divided into classes including flavones, flavonols, flavanones, flavonols, anthocyanins and isoflavonoids (Veitch & Grayer, 2011). Anthocyanins are one group of widespread natural phenolic compounds present in many flowers, fruits, and vegetables and are responsible for their orange, red, and blue colour. They are nontoxic, water-soluble compounds and are of great interest in nutrition and medicine because of their potent antioxidant capacity (Garcia-Alonso et al., 2005), ability to regulate adipocytokine gene expression (Tsuda, Ueno, Yoshikawa, Kojo, & Osawa, 2006), and therefore, possible protective effects on human health (He & Giusti, 2010; Tsuda, 2012). They are also used in dyes industry to replace synthetic pigments by natural ones (Pina, Melo, Laia, Parola, & Lima, 2012).

The interest of plant polyphenols derives from the evidence of their potent antioxidant activity and their wide range of pharmacologic properties including anti-inflammatory, antiallergic and antibacterial activities (Bravo, 1998). Furthermore, epidemiological studies have shown a correlation between increased consumption of fruits and vegetables (associated to flavonoid and other antioxidants) and reduced risk of diseases mediated by oxidative stress such as cardiovascular diseases (He, Nowson, & MacGregor, 2006; Hertog, Feskens, Hollman, Katan, & Kromhout, 1994; Hertog et al., 1995), certain types of cancer (Hertog et al., 1995). The antioxidant properties of phenolic acids and flavonoids have been related to their redox properties and chemical structures, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers. In addition, some of them display a metal

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chelation activity, which hinders transition metals from acting as oxidation promoters (Rice-Evans, Miller, & Paganga, 1997).

Exotic or unusual foods, such as the fruits of *Arbutus unedo* (strawberry-tree), *Prunus spinosa* (blackthorn), *Rosa canina* (dog rose) and *Rosa micrantha* (wild rose) may have great potential for food industries as a source of colours and flavours, as well as bioactive molecules such as phenolic compounds for dietary supplements or functional foods.

The fruits of *A. unedo* are eaten raw or made in liqueurs. Moreover, the bark or roots decoctions are used as anti-inflammatory, laxative, carminative, digestive, odontalgic and cardiotonic (Camejo-Rodrigues, 2006; Carvalho, 2010; Novais, Santos, Mendes, & Pinto-Gomes, 2004; Salgueiro, 2004). *P. spinosa* fruits are commonly eaten raw, prepared in jams or macerated with sugar, honey and brandy to obtain a digestive and laxative liqueur, which is usually drunk after copious meals; they have also been used as astringent, diuretic and purgative (Camejo-Rodrigues, 2006; Carvalho, 2010; Novais et al., 2004; Salgueiro, 2004). *R. canina* fruits are eaten raw as snacks and possess prophylactic and therapeutic activities against a wide range of ailments, including the inflammatory disorders arthritis, rheumatism, gout, colds and gastrointestinal disorders (Carvalho, 2010; Orhan, Hartevioğlu, Küpeli, & Yesilalada, 2007; Rein, Kharazmi, & Winther, 2004). Beneficial health and cosmetic properties of *R. micrantha* fruits have been transmitted and exploited by rural people for centuries; they are used to prepare homemade remedies that prevent or heal several human disorders and animal diseases (Carvalho, 2010; Parada, Carrió, Bonnet, & Vallès, 2009).

Our research group has been interested in the chemical characterisation of the mentioned wild fruits traditionally consumed in Portugal by their medicinal and edible properties. Recent phytochemical studies performed by us revealed that they have antioxidant properties and important antioxidant molecules such as tocopherols and carotenoids (Barros, Carvalho, Morais, & Ferreira, 2010; Guimarães, Barros, Carvalho, & Ferreira, 2010). A few works on the phenolic composition of *A. unedo* fruits from Italy (Pawlowska, Leo, & Braca 2006), Portugal (Fortalezas et al., 2010; Mendes, Freitas, Baptista, & Carvalho, 2011; Tavares et al., 2010) and Spain (Ganhão, Estévez, Kylli, Heinonen, & Morcuende, 2010; Pallauf, Rivas-Gonzalo, Castillo, Cano, & Pascual-Teresa, 2008), *P. spinosa* from Spain (Ganhão et al., 2010) and *R. canina* from Norway (Hvattum, 2002), Poland (Fecka, 2009), Serbia (Tumbas et al., 2012) and Spain (Ganhão et al., 2010) are available. Nevertheless, there are not available reports on phenolic composition of *R. micrantha* and of *P. spinosa* and *R. canina* samples from Portugal. In this study we performed an exhaustive characterisation of phenolic compounds present in the wild fruits of the four species.

2. Materials and methods

2.1. Samples

The fruits of *A. unedo* L. (strawberry-tree) from Ericaceae, and the Rosaceae species *P. spinosa* L. (blackthorn), *R. canina* L. (dog rose) and *R. micrantha* Borrer ex Sm. (similar to eglantine rose) were gathered in the Natural Park of Montesinho territory, in Trás-os-Montes, North-eastern Portugal. Strawberry-tree berries were collected fully ripened in November 2008; well matured blackthorn and dog rose hips were gathered in late September 2008. *R. micrantha* overripe hips that is fleshy and soft dark red fruits were collected in late autumn 2009. The ripeness degree of materials was established visually using the informant's criteria of colour and texture, according growth conditions and time of the year. In order to obtain homogeneity the colours of different materials and samples were confirmed with the Royal Horticultural Society Colour Charts Edition V.

Morphological key characters from the Flora Iberica (Castroviejo, 2001 and 2004) were used for plant identification. The fruits with seeds were lyophilized (Ly-8-FM-ULE, Snijders, Holland) and stored in the deep-freezer at −20 °C for subsequent analysis.

For each species, three samples were used and all the assays were carried out in triplicate. The results were expressed as mean ± standard deviation (SD).

2.2. Standards and reagents

HPLC-grade acetonitrile was obtained from Merck KgaA (Darmstadt, Germany). Formic, acetic and trifluoroacetic acid (TFA) were purchased from Prolabo (VWR International, France). The phenolic compounds standards were from Extrasynthèse (Genay, France). All other chemicals were of analytical grade and purchased from chemical suppliers. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

2.3. Analysis of phenolic acids, flavone/ols and flavan-3-ols

Extraction procedure: Each sample (1 g) was extracted with 30 mL of methanol:water 80:20 (*v/v*) at room temperature, 150 rpm, for 1 h. The extract was filtered through Whatman no 4 paper. The residue was then re-extracted twice with additional 30 mL portions of methanol:water 80:20 (*v/v*). The combined extracts were evaporated at 35 °C (rotary evaporator Büchi R-210) to remove methanol. For purification, the aqueous phase was deposited onto a C-18 SepPak® Vac 3 cc cartridge (Phenomenex), previously activated with methanol followed by water; sugars and more polar substances were removed by passing through 10 mL of water and the purified extract was further eluted with 5 mL of methanol. The methanolic extract was concentrated under vacuum, and re-dissolved in 1 mL of 20% aqueous methanol and filtered through a 0.22-μm disposable LC filter disk for HPLC analysis.

HPLC-DAD–ESI/MS analyses for phenolic acids and flavone/ols. The extracts were analysed using a Hewlett–Packard 1100 chromatograph (Agilent Technologies) with a quaternary pump and a diode array detector (DAD) coupled to an HP Chem Station (rev. A.05.04) data-processing station. A Waters Spherisorb S3 ODS-2 C_{18}, 3 μm (4.6 × 150 mm) column thermostatted at 35 °C was used. The solvents used were: (A) 0.1% formic acid in water, (B) acetonitrile. The elution gradient established was 10% B to 15% B over 5 min, 15–25% B over 5 min, 25–35% B over 10 min, isocratic 50% B for 10 min, and re-equilibration of the column, using a flow rate of 0.5 mL/min. Double online detection was carried out in the DAD using 280 and 370 nm as preferred wavelengths and in a mass spectrometer (MS) connected to HPLC system via the DAD cell outlet.

MS detection was performed in an API 3200 Qtrap (Applied Biosystems, Darmstadt, Germany) equipped with an ESI source and a triple quadrupole-ion trap mass analyser that was controlled by the Analyst 5.1 software. Zero grade air served as the nebulizer gas (30 psi) and turbo gas for solvent drying (400 °C, 40 psi). Nitrogen served as the curtain (20 psi) and collision gas (medium). The quadrupols were set at unit resolution. The ion spray voltage was set at −4500 V in the negative mode. The MS detector was programmed for recording in two consecutive modes: Enhanced MS (EMS) and enhanced product ion (EPI) analysis. EMS was employed to show full scan spectra, so as to obtain an overview of all of the ions in sample. Settings used were: declustering potential (DP) −450 V, entrance potential (EP) −6 V, collision energy (CE) −10 V. EPI mode was performed in order to obtain the fragmentation pattern of the parent ion(s) in the previous scan using the following parameters: DP −50 V, EP −6 V, CE −25 V, and collision

energy spread (CES) 0 V. Spectra were recorded in negative ion mode between *m/z* 100 and 1000.

The phenolic compounds present in the samples were characterised according to their UV and mass spectra and retention times compared with standards when available. For the quantitative analysis of phenolic compounds, a 5-level calibration curve was obtained by injection of known concentrations (2.5–100 μg/mL) of different standards compounds: caffeic acid ($y = 617.91x - 691.51$; $R^2 = 0.9991$); chlorogenic acid ($y = 600.27x - 763.62$; $R^2 = 0.9998$); *p*-coumaric acid ($y = 884.6x + 184.49$; $R^2 = 0.9999$); gallic acid ($y = 556.94x - 738.37$; $R^2 = 0.9988$); isorhamnetin 3-*O*-glucoside ($y = 262.31x - 9.8958$; $R^2 = 1.000$); isorhamnetin 3-*O*-rutinoside ($y = 327.42x + 313.78$; $R^2 = 0.9991$); kaempferol 3-*O*-glucoside ($y = 190.75x - 36.158$; $R^2 = 1.000$); kaempferol 3-*O*-rutinoside ($y = 175.02x - 43.877$; $R^2 = 0.9999$); quercetin 3-*O*-glucoside ($y = 316.48x - 2.9142$; $R^2 = 1.000$); quercetin 3-*O*-rutinoside ($y = 222.79x - 243.11$; $R^2 = 0.9998$), taxifolin ($y = 478.06x + 657.33$; $R^2 = 0.9987$). The results were expressed in mg per 100 g of dry weight (dw).

2.4. HPLC-DAD–ESI/MS analyses for flavan-3-ols and galloyl derivatives

The extracts were analysed using the HPLC system described above with the following conditions thermostatted at 25 °C: (A) 2.5% acetic acid in water, (B) 2.5% acetic acid/acetonitrile (90:10, v/v) and (C) HPLC-grade acetonitrile. The elution gradient established was 0–100% B for 5 min, from 0% to 5% C for 35 min, from 5% to 50% C for 5 min, isocratic 50% C for 5 min, and re-equilibration of the column, using a flow rate of 0.5 mL/min. Double online detection was carried out in the DAD using 280 nm as preferred wavelength and in a mass spectrometer (MS) connected to HPLC system via the DAD cell outlet. The MS using the same equipment described above programmed using the following settings: declustering potential (DP) −40 V, entrance potential (EP) −7 V, collision energy (CE) −20 V. EPI mode was performed in order to obtain the fragmentation pattern of the parent ion(s) in the previous scan using the following parameters: DP −40 V, EP −10 V, CE −25 V, and collision energy spread (CES) 0 V. Spectra were recorded in negative ion mode between *m/z* 100 and 1400.

The proanthocyanidins present in the samples were characterised according to their UV and mass spectra and retention times compared with standards when available. For the quantitative analysis of phenolic compounds, a 5-level calibration curve was obtained by injection of known concentrations (1–100 μg/ml) of different standards compounds: catechin ($y = 161.23x + 177.26$; $R^2 = 0.9992$); ellagic acid ($y = 36.81x + 257.13$; $R^2 = 0.9979$); gallic acid ($y = 298.26x - 634.14$; $R^2 = 0.9949$). The results were expressed in mg per 100 g of dry weight (dw).

2.5. Analysis of anthocyanins

Extraction procedure: Each sample (1 g) was extracted with 30 mL of methanol containing 0.5% TFA, and filtered through a Whatman n° 4 paper. The residue was then re-extracted twice with additional 30 mL portions of 0.5% TFA in methanol. The combined extracts were evaporated at 35 °C to remove the methanol, and re-dissolved in water. For purification, the extract solution was deposited onto a C-18 SepPak® Vac 3 cc cartridge (Phenomenex), previously activated with methanol followed by water; sugars and more polar substances were removed by passing through 10 mL of water and anthocyanin pigments were further eluted with 5 mL of methanol:water (80:20, *v/v*) containing 0.1% TFA. The methanolic extract was concentrated under vacuum, lyophilized, re-dissolved in 1 mL of 20% aqueous methanol and filtered through a 0.22-μm disposable LC filter disk for HPLC analysis.

HPLC-DAD–ESI/MS analyses: The extracts were analysed using the HPLC system described above in the conditions described by García-Marino, Hernández-Hierro, Rivas-Gonzalo, and Escribano-Bailón (2010). Separation was achieved on an AQUA® (Phenomenex) reverse phase C_{18} column (5 μm, 150 × 4.6 mm i.d) thermostatted at 35 °C. The solvents used were: (A) 0.1% TFA in water, and (B) 100% acetonitrile. The gradient employed was: isocratic 10% B for 3 min, from 10% to 15% B for 12 min, isocratic 15% B for 5 min, from 15% to 18% B for 5 min, from 18% to 30% B for 20 min and from 30% to 35% for 5 min, at a flow rate of 0.5 mL/min. Double detection was carried out by DAD, using 520 nm as the preferred wavelength, and MS using the same equipment described above. Zero grade air served as the nebulizer gas (40 psi) and turbo gas (600 °C) for solvent drying (50 psi). Nitrogen served as the curtain (100 psi) and collision gas (high). Both quadrupols were set at unit resolution. The ion spray voltage was set at 5000 V in the positive mode. EMS and ESI methods were used for acquisition of full scan spectra and fragmentation patterns of the precursor ions, respectively. Setting parameters used for EMS mode were: declustering potential (DP) 41 V, entrance potential (EP) 7.5 V, collision energy (CE) 10 V, and parameters for EPI mode were: DP 41 V, EP 7.5 V, CE 10 V, and collision energy spread (CES) 0 V.

The anthocyanins present in the samples were characterised according to their UV and mass spectra and retention times, and comparison with authentic standards when available. For quantitative analysis, a 5-level calibration curve was obtained by injection of known concentrations (50–0.25 μg/mL) of different standards compounds: cyaniding 3-*O*-glucoside ($y = 63027x - 153.83$; $R^2 = 0.9995$), delphinidin 3-*O*-glucoside ($y = 557274x + 126.24$; $R^2 = 0.9999$) and peonidin 3-*O*-glucoside ($y = 537017x - 71.469$; $R^2 = 0.9997$). The results were expressed in μg per 100 g of dry weight (dw).

3. Results

The characterisation of the phenolic compounds present in the wild fruits was performed by HPLC-DAD–MS analysis, and data of the retention time, λ_{max}, pseudomolecular ion, main fragment ions in MS^2, tentative identification and quantification of phenolic acids, flavone/ols, flavan-3-ols, and anthocyanins are presented in Tables 1–3. As an example, the HPLC profiles of flavonols (A), flavan-3-ols (B) and anthocyanins (C) of *A. unedo* fruits, recorded at 370, 280 and 520 nm, respectively, can be observed in Fig. 1.

3.1. Phenolic acids and flavone/ols

P. spinosa fruits were the only ones that presented phenolic acids, all belonging to the hydroxycinnamic acid derivative subgroup, among them, five compounds (peaks 1–4 and 6) were quinic acid derivatives identified according to their UV spectra (λ_{max} at 314–330 nm) and pseudo molecular ions $[M-H]^-$ (*m/z* at 337, 353 and 367, all of them yielding a product ion at *m/z* 191, due to the deprotonated quinic acid). Peak assignments of the different hydroxycinnamoylquinic acid isomers were made using the recommended IUPAC numbering system (IUPAC, 1976) and the hierarchical keys previously developed by Clifford, Johnston, Knight, and Kuhnert (2003, 2005). Peak 1, the major phenolic compound found in *P. spinosa* fruits, and peak 2 were identified as 3-*O*-caffeoylquinic acid and 3-*p*-coumaroylquinic acid, respectively, as peak 1 yielded deprotonated quinic acid (*m/z* at 191) as base peak and another majority ion corresponding the hydroxycinnamic acid residue at *m/z* 179 [caffeic acid-H]$^-$, and peak 2 presented *m/z* 163 [*p*-coumaric acid−H]$^-$ as base peak, a fragmentation pattern characteristic of 3-acylchlorogenic acids (Clifford et al., 2003, 2005).

Table 1
Retention time (*Rt*), wavelengths of maximum absorption in the UV–vis region (λ_{max}), pseudomolecular and MS^2 fragment ions (in brackets, relative abundances), tentative identification and quantification of the phenolic acids and flavone/ols of wild fruits.

Peak	*Rt* (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Identification type	Quantification (mg/100 g)
Arbutus unedo							
1	18.7	354	615	615(100),463(18),301(18)	Quercetin galloylhexoside derivative	DAD/MS	0.57 ± 0.03
2	18.9	352	615	615(100),463(36),301(29)	Quercetin galloylhexoside derivative	DAD/MS	1.31 ± 0.00
3	19.2	–	463	317(100)	Myricetin rhamnoside	DAD/MS	0.23 ± 0.01
4	19.5	348	609	301(100)	Quercetin 3-*O*-rutinoside	Standard/DAD/MS	1.70 ± 0.02
5	19.9	354	447	285(100)	Kaempferol hexoside	DAD/MS	0.84 ± 0.14
6	20.4	348	463	301(100)	Quercetin 3-*O*-glucoside	Standard/DAD/MS	2.34 ± 0.01
7	20.8	352	463	317(100)	Myricetin rhamnoside	DAD/MS	0.45 ± 0.01
8	23.7	352	433	301(100)	Quercetin pentoside	DAD/MS	1.32 ± 0.04
9	24.7	350	447	301(100)	Quercetin rhamnoside	DAD/MS	2.10 ± 0.04
					Total		10.86 ± 0.24
Prunus spinosa							
1	5.2	326	353	191(100),179(98),161(14),135(75)	3-*O*-Caffeoylquinic acid	Clifford et al. (2003, 2005)/DAD/MS	22.09 ± 0.11
2	7.1	312	337	191(36),163(100),155(8),119(75)	3-p-Coumaroylquinic acid	Clifford et al. (2003, 2005)/DAD/MS	0.80 ± 0.01
3	7.5	324	353	191(74),179(94),173(100),161(8),135(75)	4-*O*-Caffeoylquinic acid	Clifford et al. (2003, 2005)/DAD/MS	3.41 ± 0.03
4	8.2	326	367	193(100),191(16),173(14),149(25)	3-*O*-Feruloylquinic acid	Clifford et al. (2003, 2005)/DAD/MS	1.76 ± 0.04
5	8.5	340	401	269(100)	Apigenin pentoside	DAD/MS	1.32 ± 0.03
6	11.1	312	337	191(3),173(100),163(43),155(12),119(21)	4-p-Coumaroylquinic acid	Clifford et al. (2003, 2005)/DAD/MS	0.64 ± 0.00
7	14.3	346	739	625(100),607(7),501(9),475(7),317(14),299(24)	Myricetin derivative	DAD/MS	1.06 ± 0.05
8	15.1	328	335	179(20),161(100),135(49)	Caffeoyl hexoside	DAD/MS	1.07 ± 0.00
9	18.9	350	595	301(100)	Quercetin pentosyl-hexoside	DAD/MS	1.36 ± 0.04
10	19.5	356	609	301(100)	Quercetin rhamnosyl-hexoside	DAD/MS	2.22 ± 0.03
11	19.8	356	609	301(100)	Quercetin 3-*O*-rutinoside	Standard/DAD/MS	15.63 ± 0.33
12	20.3	352	595	301(100)	Quercetin pentosyl-hexoside	DAD/MS	6.83 ± 0.29
13	20.7	356	463	301(100)	Quercetin 3-*O*-glucoside	Standard/DAD/MS	1.36 ± 0.02
14	21.2	354	463	301(100)	Quercetin hexoside	DAD/MS	4.70 ± 0.50
15	22.9	350	593	285(100)	Kaempferol 3-*O*-rutinoside	Standard/DAD/MS	1.90 ± 0.05
16	23.8	354	609	447(4),301(100)	Quercetin hexosyl-rhamnoside	DAD/MS	7.17 ± 0.42
17	24.0	354	433	301(100)	Quercetin pentoside	DAD/MS	8.84 ± 0.26
18	24.4	358	623	315(100)	Isorhamnetin 3-*O*-rutinoside	Standard/DAD/MS	0.87 ± 0.03
19	25.1	348	447	301(100)	Quercetin rhamnoside	DAD/MS	1.30 ± 0.02
20	25.8	356	505	463(5),301(100)	Quercetin acetylhexoside	DAD/MS	1.90 ± 0.05
21	29.8	348	651	609(15),301(100)	Quercetin acetylrutinoside	DAD/MS	1.00 ± 0.01
					Total phenolic acids		29.78 ± 0.17
					Total flavone/ols		57.48 ± 0.74
					Total		87.25 ± 0.91
Rosa canina							
1	15.9	292	435	303(24),285(100)	Taxifolin pentoside	DAD/MS	1.18 ± 0.03
2	16.9	294	449	287(20),269(100),225(2),209(2),151(27)	Eriodictyol hexoside	DAD/MS	0.15 ± 0.00
3	17.6	290	449	449(100),287(44),269(64),225(14),209(14),151(55)	Eriodictyol hexoside	DAD/MS	0.50 ± 0.01
4	19.6	356	609	301(100)	Quercetin 3-*O*-rutinoside	Standard/DAD/MS	0.47 ± 0.00
5	20.0	352	477	301(100)	Quercetin glucuronide	DAD/MS	0.24 ± 0.01
6	20.4	290	435	303(26),285(100)	Taxifolin pentoside	DAD/MS	0.83 ± 0.00
7	20.5	358	463	301(100)	Quercetin 3-*O*-glucoside	Standard/DAD/MS	0.66 ± 0.01
8	20.9	352	463	301(100)	Quercetin hexoside	DAD/MS	0.78 ± 0.00
9	23.8	352	433	301(100)	Quercetin pentoside	DAD/MS	0.10 ± 0.01
10	24.2	346	623	315(100)	Isorhamnetin 3-*O*-rutinoside	Standard/DAD/MS	0.02 ± 0.01

Table 1 (*continued*)

Peak	*Rt* (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Identification type	Quantification (mg/100 g)
11	24.6	350	447	301(100)	Quercetin rhamnoside	DAD/MS	0.46 ± 0.01
12	35.4	348	593	285(100)	Kaempferol rhamnosyl-hexoside	DAD/MS	0.09 ± 0.00
					Total		5.50 ± 0.05
Rosa micrantha							
1	5.7	278	345	183(100),168(10)	Methyl gallate hexoside	DAD/MS	2.45 ± 0.02
2	16.0	294	435	303(54),285(100)	Taxifolin pentoside	DAD/MS	2.68 ± 0.01
3	17.1	296	449	449(100), 287(10),269(85),225(12),209(12),151(7)	Eriodictyol hexoside	DAD/MS	0.22 ± 0.01
4	17.8	290	449	449(100),287(44),269(66),225(12),209(25),151(44)	Eriodictyol hexoside	DAD/MS	0.63 ± 0.02
5	19.8	354	609	301(100)	Quercetin 3-*O*-rutinoside	Standard/DAD/MS	0.32 ± 0.00
6	20.3	358	477	301(100)	Quercetin glucuronide	DAD/MS	0.10 ± 0.01
7	20.6	292	435	303(53),285(100)	Taxifolin pentoside	DAD/MS	1.92 ± 0.00
8	20.7	352	463	301(100)	Quercetin 3-*O*-glucoside	Standard/DAD/MS	0.97 ± 0.01
9	21.2	352	463	301(100)	Quercetin hexoside	DAD/MS	0.90 ± 0.01
10	24.1	352	433	301(100)	Quercetin pentoside	DAD/MS	0.21 ± 0.00
11	24.4	352	623	315(100)	Isorhamnetin 3-*O*-rutinoside	Standard/DAD/MS	tr
12	25.1	346	447	301(100)	Quercetin rhamnoside	DAD/MS	0.71 ± 0.01
13	35.7	348	593	285(100)	Kaempferol rhamnosyl-hexoside	DAD/MS	0.05 ± 0.00
					Total		11.16 ± 0.04

tr-Traces.

Similarly, peak 4, with a major ion at *m/z* 193 [*p*-ferulic acid−H]$^-$, was tentatively identified as 3-*O*-feruloylquinic acid taking into account its pseudomolecular ion and fragmentation pattern. Peaks 3 and 6 were easily distinguished from its base peak at *m/z* 173 ([quinic acid−H−H_2O]$^-$), in the case of peak 3, accompanied by a secondary fragment ion at *m/z* 179 with approximately 94% abundance, which allowed their identification as 4-*O*-caffeoylquinic and 4-*p*-coumaroylquinic acids according to the fragmentation patterns described by Clifford et al. (2003, 2005). Peak 8 presented a UV spectra similar to caffeic acid, with λ_{max} around 328 nm, and a pseudo molecular ion [M−H]$^-$ at *m/z* 335, releasing a fragment at *m/z* 179 [caffeic acid−H]$^-$ (−162 mu, loss of a hexosyl moiety); it was tentatively identified as a caffeoyl hexoside.

In all the studied samples, quercetin derivatives (λ_{max} around 354 nm, and an MS^2 fragment at *m/z* 301) were particularly abundant (Table 1). Quercetin 3-*O*-rutinoside (peaks: 4- *A. unedo*, 11- *P. spinosa*, 4- *R. canina* and 5- *R. micrantha* in Table 1) and quercetin 3-*O*-glucoside (peaks: 6- *A. unedo*, 13- *P. spinosa*, 7- *R. canina* and 8- *R. micrantha*), were found in all the studied fruits. Both were positively identified according to their retention, mass and UV–vis characteristics by comparison with commercial standards. Quercetin pentoside and quercetin rhamnoside ([M−H]$^-$ at *m/z* 433 and 447, respectively) were also found in all the samples (peaks: 8 and 9- *A. unedo*; 17 and 19- *P. spinosa*; 9 and 11- *R. canina*; 10 and 12- *R. micrantha*). A quercetin hexoside ([M−H]$^-$ at *m/z* 463) was also found in *P. spinosa* (peak 14), *R. canina* (peak 8) and *R. micrantha* (peak 9) fruits. Other detected quercetin glycosides were peaks 9 and 12 in *P. spinosa*, both assigned to quercetin pentosyl-hexosides ([M−H]$^-$ at *m/z* 595); peaks 10 and 16 in *P. spinosa* ([M−H]$^-$ at *m/z* 609), tentatively identified as quercetin rhamnosyl-hexoside and quercetin hexosyl-rhamnoside; peaks 20 and 21 in *P. spinosa*, which were assigned to a quercetin acetylhexoside (pseudomolecular ion [M−H]$^-$ at *m/z* 505) and quercetin acetylrutinoside (pseudomolecular ion [M−H]$^-$ at *m/z* 651); and peaks 5 and 6 in *R. canina* and *R. micrantha* ([M−H]$^-$ at *m/z* 477), respectively, identified as quercetin glucuronides. Peaks 1 and 2 in *A. unedo* fruits were assigned to two quercetin galloylhexoside derivatives. Their identities were assigned based on their pseudomolecular ions and MS^2 spectra, releasing fragments corresponding to quercetin (*m/z* at 301) and to distinct losses of hexosyl (−162 mu), pentosyl (−132 mu), rhamnosyl (−146 mu), glucuronide (−176 mu), rutinoside (−308 mu), acetyl (−42 mu) and galloyl (−152 mu) moieties. In none of them the identity of the sugar and positions of location of the substituent could be established.

Other detected flavonols corresponded to kaempferol, isorhamnetin and myricetin derivatives. Kaempferol 3-*O*-rutinoside (peak 15 in *P. spinosa*) and isorhamnetin 3-*O*-rutinoside (peaks: 8- *P. spinosa*, 10- *R. canina* and 11- *R. micrantha*) were identified in accordance with their retention, mass spectra and UV–vis characteristics by comparison with commercial standards. Peak 5 in *A. unedo* ([M−H]$^-$ at *m/z* 447) was identified as a kaempferol hexoside, and peaks 12 and 13 in *R. canina* and *R. micrantha*, respectively, were identified as kaempferol rhamnosyl-hexosides ([M−H]$^-$ at *m/z* 593). Two myricetin rhamnosides ([M−H]$^-$ at *m/z* 463) were also found in *A. unedo* (peaks 3 and 7) releasing an MS^2 fragment at *m/z* 317 ([M−H-146]$^-$, myricetin, loss of a rhamnosyl moiety).

Peak 7 in *P. spinosa* fruits presented a λ_{max} around 346 nm, which could be associated to a flavonol, and a pseudo molecular ion [M−H]$^-$ at *m/z* 739, presenting a fragment at *m/z* 317 that could correspond to myricetin, so that it was tentatively identified as a myricetin derivative. Peak 5 in this fruit was assigned to a flavone, apigenin pentoside ([M−H]$^-$ at *m/z* 401), releasing an MS^2 fragment at *m/z* 269 ([M−H-132]$^-$, apigenin, loss of a pentosyl moiety).

Other flavonoids detected in *R. canina* and *R. micrantha* were identified as taxifolin (flavanonol) and eriodictyol (flavanone) derivatives, λ_{max} around 290 nm. Peaks 1 and 6 in *R. canina* and 2 and 7 in *R. micrantha* presented a pseudo molecular ion [M−H]$^-$ at *m/z* 435, releasing a fragment at *m/z* 303 [taxifolin-H]$^-$ (−132 mu, loss of a pentosyl moiety) and were tentatively identified as taxifolin pentosides. Peaks 2 and 3 *R. canina* and 3 and 4 in *R. micrantha* presented a pseudo molecular ion [M−H]$^-$ at *m/z* 449, releasing a fragment at *m/z* 287 [eriodictyol-H]$^-$ (−162 mu, loss of a hexosyl moiety) and were tentatively identified as eriodictyol hexosides.

P. spinosa fruits presented the highest concentration in phenolic acids (29.78 mg/100 g), especially due to 3-*O*-caffeoylquinic acid, the most abundant phenolic acid, and flavone/ols (57.48

Table 2
Retention time (*Rt*), wavelengths of maximum absorption in the UV–vis region (λ_{max}), pseudomolecular and MS^2 fragment ions (in brackets, relative abundances), tentative identification and quantification of flavan-3-ols and galloyl derivatives in wild fruits.

Peak	*Rt* (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Identification type	Quantification (mg/100 g)
Arbutus unedo							
1	9.9	274	343	191 (100),169 (13)	Galloylquinic acid	Pawlowska et al. (2006)/Mendes et al. (2011) and Tavares et al. (2010)/DAD/MS	8.65 ± 0.00
2	11.3	278	331	169 (100)	Galloylhexoside acid	DAD/MS	1.02 ± 0.03
3	12.5	276	593	423(75),407(25),305 (100)	PA dimer (GC + C)[b]	DAD/MS	1.07 ± 0.00
4	12.9	276	325	169 (100)	Galloyl shikimic acid	Pawlowska et al. (2006)/Mendes et al. (2011) and Tavares et al. (2010)/DAD/MS	3.61 ± 0.01
5	15.7	274	577	451(17),425(26),407(100), 289(78), 287(7)	B1 dimer	DAD/MS	8.56 ± 0.31
6	16.4	280	865	865(100),739(9), 713(9), 695(17), 577(9), 575(9), 425(12), 407(17), 289(12),287(12)	B-type procyanidin trimer	DAD/MS	4.11 ± 0.14
7	17.5	278	865	865(100),739(3), 713(3), 695(2), 577(11), 575(3), 425(4), 407(5), 289(9),287(7)	B-type procyanidin trimer	DAD/MS	2.76 ± 0.07
8	18.3	278	289	245 (70), 205 (33), 151 (21), 137 (33)	(+)-catechin	Standard/DAD/MS	13.51 ± 0.93
9	18.4	278	495	343(42),191(100)	Digalloylquinic acid	Pawlowska et al. (2006)/Mendes et al. (2011) and Tavares et al. (2010)/DAD/MS	3.49 ± 0.42
10	19.4	278	1153	865 (7), 577 (8), 289 (100)	B-type procyanidin tetramer	DAD/MS	2.94 ± 0.07
11	22.4	278	577	451(17),425(75),407(100), 289(58), 287(17)	B-type procyanidin dimer	DAD/MS	3.35 ± 0.06
12	22.9	276	477	325(100),169(29)	Digalloylquinic shikimic acid	Pawlowska et al. (2006)/Mendes et al. (2011) and Tavares et al. (2010)/DAD/MS	1.81 ± 0.04
13	23.9	270	633	633(100),463(12),301(70),275(5)	Strictinin ellagitannin	Pawlowska et al. (2006)/Mendes et al. (2011) and Tavares et al. (2010)/DAD/MS	3.54 ± 0.01
14	28.3	270	477	325(100),169(54)	Digalloylquinic shikimic acid	Pawlowska et al. (2006)/Mendes et al. (2011) and Tavares et al. (2010)/DAD/MS	2.51 ± 0.00
					Total flavan-3-ols		36.30 ± 0.70
					Total galloyl derivatives		24.63 ± 0.42
					Total		60.93 ± 0.27
Rosa canina							
1	11.5	280	1351	1019(9),899(26),675(100),451(96),287(9)	PA trimer triglycoside	DAD/MS	0.77 ± 0.00
2	12.3	280	1189	1189(100), 898(23),739(17),575(10),449(17),289(7)	PA trimer diglycoside	DAD/MS	1.37 ± 0.00
3	12.9	280	819[a]	739(100),674(2),289(15)	PA tetramer triglycoside	Salminen et al. (2005)/ DAD/MS	0.69 ± 0.04
4	13.5	274	819[a]	739(38),674(2),289(100)	PA tetramer triglycoside	Salminen et al. (2005)/ DAD/MS	1.21 ± 0.07
5	14.9	280	739	449(18),425(29),407(100),289(47)	PA dimer monoglycoside	DAD/MS	3.27 ± 0.10
6	15.6	280	451	289(100)	(+)-Catechin-hexoside	DAD/MS	1.62 ± 0.06
7	15.9	272	577	451(4),425(52),407(100),289(69)	Procyanidin dimer B1	DAD/MS	1.68 ± 0.09
8	16.3	278	577	425(31), 407(100), 289(25)	Procyanidin dimer B3	DAD/MS	1.50 ± 0.13
9	17.7	282	865	865(100),695(10), 577(50), 449(15), 407(25), 287(35)	B-type procyanidin trimer	DAD/MS	0.74 ± 0.08
10	17.9	280	739	739(100),449(11),425(11),407(48),289(22)	Procyanidin dimer monoglycoside	DAD/MS	1.32 ± 0.02
11	18.7	278	289	289(100),245(29), 205(8), 179(7), 137(4)	(+)-Catechin	Standard/ DAD/MS	3.59 ± 0.17
12	30.1	280	865	865(100),739(14),577(21),575(21),425(14),407(14),289(21)	B-type procyanidin trimer	DAD/MS	2.14 ± 0.08
					Total flavan-3-ols		19.90 ± 0.51
Rosa micrantha							
1	11.3	280	1351	1019(23),899(26),675(100),451(73),287(10)	PA trimer triglycoside	DAD/MS	2.32 ± 0.05
2	12.1	280	1189	898(37),739(19),575(14),449(22),289(100)	PA trimer diglycoside	DAD/MS	2.02 ± 0.04
3	12.7	280	819[a]	819(100),739(18),674(4),289(35)	PA tetramer triglycoside	Salminen et al. (2005)/ DAD/MS	0.88 ± 0.07
4	13.2	280	819[a]	739(18),674(27),289(100)	PA tetramer triglycoside	Salminen et al. (2005)/ DAD/MS	2.52 ± 0.06
5	13.8	280	1351	1019(13),899(13),675(50),451(100),287(14)	PA trimer triglycoside	DAD/MS	0.49 ± 0.01

Table 2 (*continued*)

Peak	*Rt* (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Identification type	Quantification (mg/100 g)
6	14.5	280	739	739(100),449(27),425(13),407(63),289(43)	PA dimer monoglycoside	DAD/MS	4.93 ± 0.06
7	15.3	280	451	289(100)	(+)-catechin-hexoside	DAD/MS	2.64 ± 0.14
8	15.6	274	577	451(15),425(55),407(100),289(91)	Procyanidin dimer B1	DAD/MS	1.69 ± 0.04
9	17.6	280	739	739(100),449(25),425(15),407(47),289(22)	Procyanidin dimer monoglycoside	DAD/MS	0.94 ± 0.08
10	18.4	278	289	289(100),245(72), 205(32), 179(16), 137(18)	(+)-catechin	Standard/ DAD/MS	2.90 ± 0.06
11	19.8	284	819[a]	819(100),739(33),674(11),289(67)	PA tetramer triglycoside	Salminen et al. (2005)/ DAD/MS	1.54 ± 0.07
12	19.9	280	865	865(100),739(15),577(3),575(5),425(2),407(5),289(9)	B-type procyanidin trimer	DAD/MS	0.68 ± 0.12
13	21.6	280	851	851(100),739(6), 671(4), 561(62), 537(17), 407(10), 381(33), 357(6), 289(12)	PA trimer (2C + A)[b]	DAD/MS	1.80 ± 0.06
14	25.9	282	739	739(100),449(11),425(33),407(11),289(33)	PA dimer monoglycoside	DAD/MS	1.98 ± 0.11
15	26.8	280	851	851(100),739(6), 671(6), 561(71), 537(18),407(12),381(65), 357(12), 289(12)	PA trimer (2C + A)[b]	DAD/MS	1.40 ± 0.08
16	28.8	280	1027	1027(100),900(4),737(3),575(4),407(15),287(7)	PA trimer monoglycoside	DAD/MS	0.96 ± 0.34
17	29.9	282	865	865(100),739(6),577(25),575(31),425(13),407(25),289(25)	B-type procyanidin trimer	DAD/MS	1.63 ± 0.03
18	35.6	276	577	451(14),425(51),407(71),289(100)	B-type procyanidin dimer	DAD/MS	1.31 ± 0.03
					Total flavan-3-ols		32.62 ± 0.70

[a] $[M-H]2^-$.
[b] C: (epi)catechin, A: (epi)afzelechin, GC: (epi)gallocatechin.

Table 3
Retention time (*Rt*), wavelengths of maximum absorption in the UV–vis region (λ_{max}), pseudomolecular and MS^2 fragment ions (in brackets, relative abundances), tentative identification and quantification of the anthocyanins of wild fruits.

Peak	*Rt* (min)	λ_{max} (nm)	Molecular ion $[M+H]^+$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Identification type	Quantification (μg/100 g dw)
Arbutus unedo							
1	18.0	518	465	303(100)	Delphinidin 3-*O*-glucoside	Standard/DAD/MS	0.91 ± 0.01
2	22.4	518	449	287(100)	Cyanidin 3-*O*-glucoside	Standard/DAD/MS	11.40 ± 0.03
3	25.9	518	419	287(100)	Cyanidin 3-*O*-pentoside	Standard/DAD/MS	1.45 ± 0.02
					Total		13.77 ± 0.01
Prunus spinosa							
1	23.1	518	449	287(100)	Cyanidin 3-*O*-glucoside	Standard/DAD/MS	19.83 ± 0.05
2	24.4	518	595	449(6), 287(100)	Cyanidin 3-*O*-rutinoside	Standard/DAD/MS	31.12 ± 0.11
3	30.9	518	463	301(100)	Peonidin 3-*O*-glucoside	Standard/DAD/MS	10.73 ± 0.16
4	31.4	520	609	463(3), 301(100)	Peonidin 3-*O*-rutinoside	Standard/DAD/MS	34.47 ± 0.03
5	32.2	518	419	287(100)	Cyanidin 3-*O*-pentoside	Standard/DAD/MS	1.49 ± 0.12
6	37.4	520	433	301(100)	Peonidin 3-*O*-pentoside	Standard/DAD/MS	0.26 ± 0.03
7	38.2	520	491	287(100)	Cyanidin 3-*O*-acetylglucoside	DAD/MS	1.77 ± 0.01
8	42.3	520	505	301(100)	Peonidin 3-*O*-acetylglucoside	DAD/MS	0.73 ± 0.05
					Total		100.40 ± 0.47
Rosa canina							
1	22.6	516	449	287(100)	Cyanidin 3-*O*-glucoside	Standard/DAD/MS	0.68 ± 0.01
Rosa micrantha							
1	22.5	516	449	287(100)	Cyanidin 3-*O*-glucoside	Standard/DAD/MS	1.19 ± 0.03

mg/100 g), in which quercetin 3-*O*-rutinoside (15.63 mg/100 g) was the main compound. *A. unedo* fruits presented quercetin 3-*O*-glucoside as the majority flavonol (2.34 mg/100 g).

Taxifolin pentoside was the majority flavanonol derivative in *R. canina* and *R. micrantha* (1.18 and 2.68 mg/100 g, respectively). *R. micrantha* (11.16 mg/100 g) presented higher amount of total flavonoids (flavonols, flavanone and flavanonol) compared to *R. canina* (5.50 mg/100 g).

3.2. Flavan-3-ols and galloyl derivatives

Flavan-3-ols (i.e., catechins and proanthocyanidins) were other relevant flavonoids found in *A. unedo*, *R. canina* and *R. micrantha* fruits. Peak 8 in *A. unedo*, peak 11 in *R. canina* and peak 10 in *R. micrantha* were identified as (+)-catechin by comparison of its UV spectra and retention time with a commercial standard. Peak 6 in *R. canina* and Peak 7 in *R. micrantha* presented a pseudomolecular ion $[M-H]^-$ at *m/z* 451, releasing an MS^2 fragment at *m/z* 289 ($[M-H-162]^-$, loss of an hexosyl moiety), corresponding to a catechin monomer. This compound was tentatively identified as (+)-catechin-*O*-hexoside, identity that was coherent with its earlier elution (higher polarity) compared with its parent aglycone. Peak 5 in *A. unedo*, peak 7 in *R. canina* and peak 8 in *R. micranta* were identified as procyanidin dimer B1 by comparison of its UV spectra and retention time with a standard available in the laboratory. Similarly, peak 8 in *R. canina* was identified as procyanidin dimer B3. Other signals at *m/z* 577, 865 and 1153, in *A. unedo* (peaks 6, 7, 10 and 11), *R. canina* (peaks 9 and 12) and *R. micrantha* (peaks 12, 17 and 18) can be respectively associated to B-type procyanidin dimers, trimers and tetramers (*i.e.*, (epi)catechin units with C4–C8

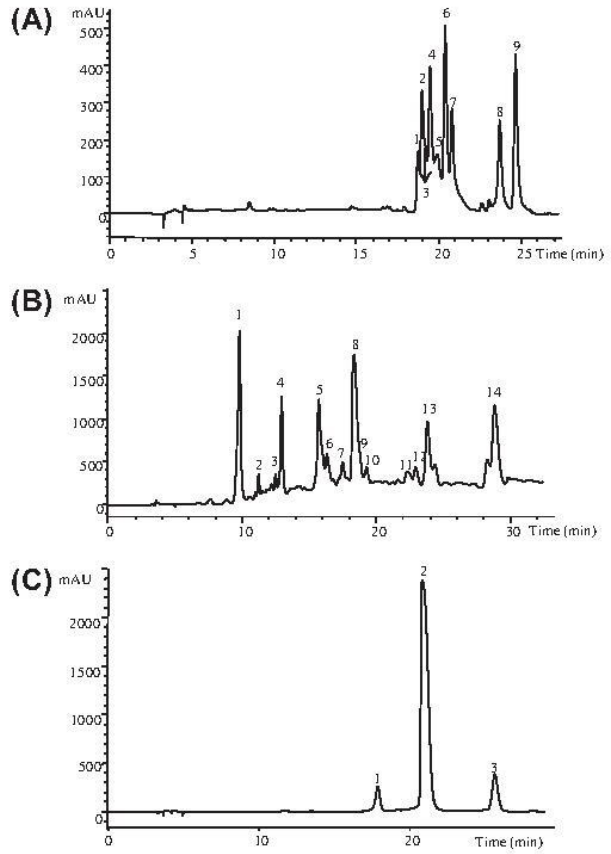

Fig. 1. HPLC phenolic profiles of *Arbutus unedo* obtained at 370 nm (A), 280 nm (B) and 520 nm (C) used for recording flavone/ols, flavan-3-ols and galloyl derivatives, and anthocyanins, respectively. Note that different chromatographic conditions were used in each case.

or C4–C6 interflavonoid linkages). Peak 3 in *A. unedo* (pseudomolecular ion $[M-H]^-$ at *m/z* 593) was coherent with a proanthocyanidin dimer consisting of one (epi)gallocatechin unit and one (epi)catechin unit. Peaks 13 and 15 in *R. micrantha* ($[M-H]^-$ at *m/z* 851) were coherent with proanthocyanidin trimers formed by two (epi)catechin units and one (epi)afzelechin unit. Pseudomolecular ions $[M-H]^-$ at *m/z* 739, 1027, 1189 and 1351 in *R. canina* (peaks 1, 2, 5 and 10) and in *R. micrantha* (peaks 1, 2, 5, 6, 9 and 16) could correspond to procyanidin dimers or trimers having one to three sugar units attached to them. Also, peaks corresponding to double-charged deprotonated ions $[M-2H]^{2-}$ at *m/z* 819 were detected in *R. canina* (peaks 3 and 4) and *R. micrantha* (peaks 4, 5 and 11), which could be interpreted as procyanidin tetramers bearing three hexosyl residues in accordance with previous observations by Salminen et al. (2005).

Other compounds detected in the chromatograms obtained for flavan-3-ol analysis were identified as different galloyl derivatives according to their pseudomolecular ions and MS^2 fragmentation behaviour (Table 2). Peaks 1, 2, 4, in *A. unedo* presented pseudomolecular ions $[M-H]^-$ at *m/z* 343, 331 and 325, respectively, releasing a fragment at *m/z* 169 [gallic acid–H]$^-$ (−174, −162 and −156 mu, losses of quinic acid, hexosyl and shikimic acid moieties). These compounds were tentatively identified as galloylquinic acid, galloylhexoside and galloylshikimic acid. Other signals at *m/z* 495 and 477 in *A. unedo* (peaks 9, 12 and 14) can be respectively associated to digalloylquinic and digalloylquinic shikimic acids, respectively. These galloyl derivatives were already described in *A. unedo* by by Pawlowska et al. (2006), Mendes et al. (2011) and Tavares et al. (2010).

Peak 13 in *A. unedo* showed a spectra UV similar to ellagic acid, and was identified as an ellagitannin. This compound was assigned as strictinin ellagitannin according to its pseudomolecular ion ($[M-H]^-$ at *m/z* 633) and fragmentation pattern as described by Fortalezas et al. (2010), Tavares et al. (2010) and Mendes et al. (2011).

Peak 1 in *R. micrantha* (Table 1) presented λ_{max} around 278 nm and was identified as methyl gallate hexoside ($[M-H]^-$ at *m/z* 345). A similar compound was already reported in rose hip extracts by Hvattum (2002) and further identified by Fecka (2009) as methylgallate 3-*O*-β-glucoside.

(+)-Catechin was the most abundant flavan-3-ol found in *A. unedo* (13.51 mg/100 g) and *R. canina* (3.59 mg/100 g), and also was a prominent compound in *R. micrantha* (2.90 mg/100 g), although a glycosylated proanthocyanidin dimer (peak 6, 4.93 mg/100 g) was the most abundant flavan-3-ol in this fruit. *A. unedo* fruits presented the highest concentration in proanthocyanidins (36.30 mg/100 g dw).

3.3. Anthocyanins

The anthocyanin profile obtained for *P. spinosa* fruits, consisting of eight compounds, was more complex that those found in the other fruits. Only one anthocyanin was detected in *R. canina* and *R. micrantha* fruits, whereas three anthocyanins were detected in *A. unedo* fruits. The analytical characteristics, identities and concentrations of the anthocyanins found in the different samples are presented in Table 3. Cyanidin 3-*O*-glucoside, peonidin 3-*O*-glucoside and delphinidin 3-*O*-glucoside were positively identified by comparison with standards. The identity of cyaniding 3-*O*-rutinoside and peonidin 3-*O*-rutinoside were also confirmed by comparison of their chromatographic and UV and mass spectral characteristics with data in our library. Peaks 5 and 6 of *P. spinosa* and peak 3 of *A. unedo* were assigned as cyaniding 3-*O*-pentoside and peonidin 3-*O*-pentoside, respectively, based on their mass spectra, which showed an MS^2 signal at *m/z* 287 (cyanidin; $[M-132]^+$, loss of a pentosyl moiety) and *m/z* 301 (peonidin; $[M-132]^+$, loss of a pentosyl moiety). Peaks 7 and 8 presented pseudomolecular ions $[M-H]^+$ at *m/z* 491 and 505 releasing MS^2 fragments at *m/z* 287 and 301 (cyanidin and peonidin, respectively; $[M-H-42-162]^+$, loss of an acetylhexoside moiety), and were tentatively identified as cyaniding 3-*O*-acetylglucoside and peonidin 3-*O*-acetylglucoside, respectively. Cyanidin 3-*O*-glucoside was found in all the samples, being the major anthocyanin in most of them, with exception of *P. spinosa* fruits in which cyaniding 3-*O*-rutinoside and peonidin 3-*O*-rutinoside were the major anthocyanins. These fruits also presented the highest anthocyanin concentrations, which was coherent with their higher pigmentation.

4. Discussion

The chemopreventive effect of polyphenols has often been associated with their antioxidant activity. For example, chlorogenic acids are responsible for beneficial roles of some medicinal plants in the prevention of certain oxidative diseases (Morishita & Ohnishi, 2001). Quercetin is also a very efficient antioxidant (Pietta, 2000) and appears to be active in many diseases such as cancer (Choi et al., 2001), cardiovascular (Yoshizumi et al., 2001), and neurodegenerative (Schroeter, Spencer, Rice-Evans, & Williams, 2001) disorders. Derivatives of both these compounds were abundant in the studied fruits.

A range of health related properties are also reported for flavon-3-ols namely antiviral (Shahat et al., 2002), insulin-like (Anderson et al., 2004), antitumor (Miura et al., 2008), anti-inflammatory

(Terra et al., 2007), and antioxidant (Maldonado, Rivero-Cruz, Mata, & Pedraza-Chaverri, 2005) activities. B-type trimer showed higher antioxidant activity compared to higher oligomers (Shahat et al., 2002). Compared to resveratrol and ascorbic acid, an A-type procyanidin dimer and trimer had similar or better radical scavenging abilities (Maldonado et al., 2005). Thus, both A- and B-type procyanidins have been reported to exert antioxidant activity.

Anthocyanins have demonstrated that, in addition to their colourful characteristics, they possess some positive therapeutic effects, mainly linked with their strong antioxidant properties (Wang, Cao, & Prior, 1997). Cyanidin-3-*O*-glucoside (kuromanin) has been found to have the highest oxygen radical absorbance capacity (Wang et al., 1997). This compound is the most ubiquitous and it represents the main anthocyanin in the edible parts of several plants (Dugo, Mondello, Errante, Zappia, & Dugo, 2001), being also found in the four fruits studied herein.

The highest phenolic acids (29.78 mg/100 g), flavone/ols (57.48 mg/100 g) and anthocyanins (100.40 μg/100 g) contents were found in *P. spinosa* fruits, despite no flavan-3-ols compounds were detected in this sample.

As far as we know, there is no information on the phenolic composition of *R. micrantha* fruits; our group has already characterised the phenolic compounds of flowers from this species (Barros et al., 2013). The total amounts of each phenolic group has been reported for *P. spinosa* fruits from Spain (Ganhão et al., 2010), but the individual profile of this species has not been previously described. Ganhão et al. (2010) also analysed total phenolic compounds in *A. unedo* and *R. canina*. Otherwise, for *A. unedo* and *R. canina*, there are a few publications on their individual profile of phenolic composition but from other countries (Fecka, 2009; Fortalezas et al., 2010; Hvattum, 2002; Mendes et al., 2011; Pallauf et al., 2008; Pawlowska et al., 2006; Tavares et al., 2010; Tumbas et al., 2012).

R. canina fruits from Portugal presented some similarities in the phenolic composition to samples from Norway, Poland and Serbia (Fecka, 2009; Hvattum, 2002; Tumbas et al., 2012). Tumbas et al. (2012) identified in the Serbian sample different phenolic acids (gallic, protocatechuic, caffeic, syringic, coumaric, vanillic, ferulic and ellagic acids) and flavonoid aglycones, such as quercetin, kaempferol and myricetin. In the Portuguese sample these types of flavonols were found but all of them were glycosylated; this could be due to the fact that the extract studied by Tumbas et al. (2012) was an infusion of rosehips, and hydrolyses of the sugar moieties could have occurred. The sample from Serbia presented a lower concentration in phenolic compounds and quercetin (296.5 μg/kg of rosehip tea) was the major compound. As for the samples studied herein, (+)-catechin (3.59 mg/100 g dw) was the major phenolic compound found. Samples of *R. canina* from Poland (Fecka, 2009) presented a higher concentration of phenolic compounds than the one studied here, being methylgallate-3-*O*-β-glucoside and catechin the main phenolic compounds. Furthermore, Fecka (2009) identified the presence of ellagitannins such as tellimagrandin I and II and rugosin A, B, D and E, which were not detected in our study. No quantitative information was provided in the study described by Hvattum (2002) regarding phenolic compounds of *R. canina* sample from Norway. Nevertheless, its phenolic profile was similar to the Portuguese sample here analysed, despite some differences in the identification of flavan-3-ols.

For *A. unedo* fruits there are three different studies on the individual phenolic profile of samples from Italy, Spain and Portugal (Fortalezas et al., 2010; Mendes et al., 2011; Pallauf et al., 2008; Pawlowska et al., 2006; Tavares et al., 2010) and they were all very similar to the one obtained in this work, with exception of Italian *A. unedo* fruits (Pawlowska et al., 2006), for which only gallic acid derivatives and anthocyanins were reported, but not flavonols. The comparison of phenolic compounds quantification was not possible, due to the fact that these authors only quantified the anthocyanins, expressing the results in fresh weight. However, the amounts obtained for Spanish *A. unedo* fruits (Pallauf et al., 2008) were very similar to ours. Mendes et al., 2011; Tavares et al., 2010 and Fortalezas et al., 2010 studied a different sample from Portugal, but did not present any type of quantification.

Previous *in vitro* chemical and biochemical assays demonstrated that the studied fruits have a high antioxidant activity and that this could be correlated to their phenolic composition (Barros, Carvalho, & Ferreira 2011; Barros et al., 2010; Guimarães et al., 2010).

Overall, *P. spinosa* fruits presented the highest concentration in phenolic acids and flavone/ols, being 3-*O*-caffeoylquinic acid and quercetin 3-*O*-rutinoside the major compounds. (+)-Catechin was the most abundant compound in *A. unedo* and *R. canina* fruits. *A. unedo* fruits presented the highest concentration in flavan-3-ols. Cyanidin 3-*O*-glucoside was found in all the studied fruits, being the major anthocyanin in most of them, with the exception of *P. spinosa*, were cyaniding 3-*O*-rutinoside and peonidin 3-*O*-rutinoside predominated. All in all, *P. spinosa* presented the highest levels of phenolic acids and flavonoids, including anthocyanins, flavonols and flavones, although no flavan-3-ols could be identified in its fruits. The present study represents a contribution to the chemical characterisation of phenolic extracts from wild fruits with reported antioxidant activity and traditionally used for several medicinal applications. The studied fruits may have great potential for food industries as a source of colours and flavours, as well as bioactive molecules such as phenolic compounds for dietary supplements or functional foods.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2013.06.071.

References

Anderson, R. A., Broadhurst, C. L., Polansky, M. M., Schmidt, W. F., Khan, A., Flanagan, V. P., et al. (2004). Isolation and characterization of polyphenol type-A polymersfrom cinnamon with insulin-like biological activity. *Journal of Agricultural and Food Chemistry, 52*, 65–70.

Barros, L., Alves, C. T., Dueñas, M., Silva, S., Oliveira, R., Carvalho, A. M., et al. (2013). Towards antifungal compounds exploring phenolic extracts of wild medicinal flowers from Portugal. *Industrial Crops and Products, 2013*(44), 104–110.

Barros, L., Carvalho, A. M., & Ferreira, I. C. F. R. (2011). Exotic fruits as a source of important phytochemicals: Improving the traditional use of *Rosa canina* fruits in Portugal. *Food Reseach International, 44*, 2233–2236.

Barros, L., Carvalho, A. M., Morais, J. S., & Ferreira, I. C. F. R. (2010). Strawberry-tree, blackthorn and rose fruits: Detailed characterization in nutrients and phytochemicals with antioxidant properties. *Food Chemistry, 120*, 247–254.

Bravo, L. (1998). Polyphenols: Chemistry, dietary sources, metabolism, and nutritional significance. *Nutrition Reviews, 56*, 317–333.

Camejo-Rodrigues, J. S. (2006). *Recolha dos 'Saber-Fazer' Tradicionais das Plantas Aromáticas e Medicinais, Concelhos de Aljezur*. Associação Aflosul, Bordeira: Lagos e Vila do Bispo.
Carvalho, A. M. (2010). Plantas y sabiduría popular del Parque Natural de Montesinho. *Un estudio etnobotánico en Portugal. Biblioteca de Ciencias 35*. Madrid: Consejo Superior de Investigaciones Científicas.
Castroviejo, S.(coord). (2001). Flora Iberica. Plantas vasculares de la Península Ibérica e Islas Baleares. *Rosaceae* (Vol. VI). Real Jardín Botánico, Madrid: CSIC.
Castroviejo, S.(coord). (2004). *Flora Iberica. Plantas vasculares de la Península Ibérica e Islas Baleares* (Vol. IV). Real Jardín Botánico, Madrid: CSIC.
Choi, J. A., Kim, J. Y., Lee, J. Y., Kang, C. M., Kwon, H. J., Yoo, Y. D., et al. (2001). Induction of cell cycle arrest and apoptosis in human breast cancer cells by quercetin. *International Journal of Oncology, 19*, 837–844.
Clifford, M. N., Johnston, K. L., Knight, S., & Kuhnert, N. A. (2003). A hierarchical scheme for LC–MSn identification of chlorogenic acids. *Journal of Agriculture and Food Chemical, 51*, 2900–2911.
Clifford, M. N., Knight, S., & Kuhnert, N. A. (2005). Discriminating between the six isomers of dicaffeoylquinic acid by LC–MSn. *Journal of Agriculture and Food Chemical, 53*, 3821–3832.
Dugo, P., Mondello, L., Errante, G., Zappia, G., & Dugo, G. (2001). Identification of anthocyanins in berries by narrow-bore high performance liquid chromatography with electrospray ionization detection. *Journal of Agricultural and Food Chemistry, 49*, 3987–3992.
Fecka, I. (2009). Qualitative and quantitative determination of hydrolysable tannins and other polyphenols in herbal products from meadowsweet and dog rose. *Phytochemical Analysis, 20*, 177–190.
Fortalezas, S., Tavares, L., Pimpao, R., Tyagi, M., Pontes, V., Alves, P. M., et al. (2010). Antioxidant properties and neuroprotective capacity of strawberry tree fruit (*Arbutus unedo*). *Nutrients, 2*, 214–229.
Ganhão, R., Estévez, M., Kylli, P., Heinonen, M., & Morcuende, D. (2010). Characterization of selected wild mediterranean fruits and comparative efficacy as inhibitors of oxidative reactions in emulsified raw pork burger patties. *Journal of Agriculture and Food Chemical, 58*, 8854–8861.
Garcia-Alonso, M., Rimbach, G., Sasai, M., Nakahara, M., Matsugo, S., Uchida, Y., et al. (2005). Electron spin resonance spectroscopy studies on the free radical scavenging activity of wine anthocyanins and pyranoanthocyanins. *Molecular Nutrition and Food Research, 49*, 1112–1119.
García-Marino, M., Hernández-Hierro, J. M., Rivas-Gonzalo, J. C., & Escribano-Bailón, M. T. (2010). Colour and pigment composition of red wines obtained from co-maceration of Tempranillo and Graciano varieties. *Analytical Chimica Acta, 660*, 134–142.
Guimarães, R., Barros, L., Carvalho, A., & Ferreira, I. C. F. R. (2010). Studies on chemical constituents and bioactivity of *Rosa micrantha*: An alternative antioxidants source for food, pharmaceutical, or cosmetic applications. *Journal of Agriculture and Food Chemical, 58*, 6277–6284.
He, J., & Giusti, M. (2010). Anthocyanins: Natural colorants with health-promoting propertie. *Annual Review of Food Science and Technology, 1*, 163–187.
He, F. J., Nowson, C. A., & MacGregor, G. A. (2006). Fruit and vegetable consumption and stroke: Metaanalysis of cohort studies. *Lancet, 367*, 320–326.
Hertog, M. G. L., Feskens, E. J. M., Hollman, P. C. H., Katan, M. B., & Kromhout, D. (1994). Dietary flavonoids and cancer risk in the Zutphen elderly study. *Nutrition and Cancer, 22*, 175–184.
Hertog, M. G. L., Kromhout, D., Aravanis, C., Blackburn, H., Buzina, R., Fidanza, F., et al. (1995). Flavonoid intake and long-term risk of coronary heart disease and cancer in the seven countries study. *Archives of Internal Medicine, 155*, 381–386.
Hvattum, E. (2002). Determination of phenolic compounds in rose hip (*Rosa canina*) using liquid chromatography coupled to electrospray ionization tandem mass spectrometry and diode-array detection. *Rapid Communications in Mass Spectrometry, 16*, 655–662.
IUPAC (1976). Nomenclature of cyclitols. *Biochemical Journal, 153*, 23–31.
Maldonado, P. D., Rivero-Cruz, I., Mata, R., & Pedraza-Chaverri, J. (2005). Antioxidant activity of A-type proanthocyanidins from *Geranium niveum* (Geraniaceae). *Journal of Agricultural and Food Chemistry, 53*, 1996–2001.
Mendes, L., Freitas, V., Baptista, P., & Carvalho, M. (2011). Comparative antihemolytic and radical scavenging activities of strawberry tree (*Arbutus unedo* L.) leaf and fruit. *Food and Chemical Toxicology, 49*, 2285–2291.
Miura, T., Chiba, M., Kasai, K., Nozaka, H., Nakamura, T., Shoji, T., et al. (2008). Apple procyanidins induce tumor cell apoptosis through mitochondrial pathway activation of caspase-3. *Carcinogenesis, 29*, 585–593.
Morishita, H., & Ohnishi, M. (2001). Absorption, metabolism and biological activities of chlorogenic acids and related compounds. *Bioactive Natural Products, 25*(Part F), 919–953.
Novais, H. M., Santos, I., Mendes, S., & Pinto-Gomes, C. (2004). Studies on pharmaceutical ethnobotany in Arrábida Natural Park. *Journal of Ethnopharmacology, 93*, 183–195.
Orhan, D. D., Hartevioğlu, A., Küpeli, E., & Yesilalada, E. (2007). *In vivo* anti-inflammatory and antinociceptive activity of the crude extract and fractions from *Rosa canina* L. fruits. *Jornal of Ethnopharmacology, 112*, 394–400.
Pallauf, K., Rivas-Gonzalo, J. C., Castillo, M. D., Cano, M. P., & Pascual-Teresa, S. (2008). Characterization of the antioxidant composition of strawberry tree (*Arbutus unedo* L.) fruits. *Journal of Food Composition and Analysis, 21*, 273–281.
Parada, M., Carrió, E., Bonnet, M. A., & Vallès, J. (2009). Ethnobotany of the Alt Empordà region (Catalonia, Iberian Peninsula). Plants used in human traditional medicine. *Jornal of Ethnopharmacology, 124*, 609–618.
Pawlowska, A. M., Leo, M., & Braca, A. (2006). Phenolics of *Arbutus unedo* L. (Ericaceae) fruits: Identification of anthocyanins and gallic acid derivatives. *Journal of Agricultural and Food Chemistry, 54*, 10234–10238.
Pietta, P. G. (2000). Flavonoids as antioxidants. *Journal of Natural Products, 63*, 1035–1042.
Pina, F., Melo, M. J., Laia, C. A. T., Parola, A. J., & Lima, J. C. (2012). Chemistry and applications of flavylium compounds: A handful of colours. *Chemical Society Reviews, 41*, 869–908.
Rein, E., Kharazmi, A., & Winther, K. (2004). A herbal remedy, Hyben Vital (stand. powder of a subspecies of *Rosa canina* fruits), reduces pain and improves general wellbeing in patients with osteoarthritis – A double-blind, placebo-controlled, randomised trial. *Phytomedicine, 11*, 383–391.
Rice-Evans, C. A., Miller, N. J., & Paganga, G. (1997). Antioxidant properties of phenolic compounds. Review. *Trends in Plant Science, 2*, 152–159.
Salgueiro, J. (2004). *Ervas, usos e saberes*. Lisboa: MARCA, Associação de Desenvolvimento Local.
Salminen, J. P., Karonen, M., Lempa, K., Liimatainen, J., Sinkkonen, J., Lukkarinen, M., et al. (2005). Characterisation of proantocyanidin aglycones and glycosides from rose hips by high-performance liquid chromatography–mass spectrometry, and their rapid quantification together with Vitamin C. *Journal of Chromatography A, 1077*, 170–180.
Schroeter, H., Spencer, J. P. E., Rice-Evans, C., & Williams, R. J. (2001). Flavonoids protect neurons from oxidized low-density-lipoprotein-induced apoptosis involving c-Jun N-terminal kinase (JNK), c-Jun and caspase-3. *Biochemical Journal, 358*, 547–557.
Shahat, A. A., Cos, P., De Bruyne, T., Apers, S., Hammouda, F. M., Ismail, S. I., et al. (2002). Antiviral and antioxidant activity of flavonoids and proanthocyanidins from *Crataegus sinaica*. *Planta Medica, 68*, 539–541.
Tavares, L., Fortalezas, S., Carrilho, C., McDougall, G. J., Stewart, D., Ferreira, R. B., et al. (2010). Antioxidant and antiproliferative properties of strawberry tree tissues. *Journal of Berry Research, 1*, 3–12.
Terra, X., Valls, J., Vitrac, X., Merrillon, J. M., Arola, L., Ardevol, A., et al. (2007). Grape-seed procyanidins act as anti-inflammatory agents in endotoxin-stimulated RAW 264.7 macrophages by inhibiting NFkB signalling pathway. *Journal of Agricultural and Food Chemistry, 55*, 4357–4365.
Tsuda, T. (2012). Dietary anthocyanin-rich plants: Biochemical basis and recent progress in health benefits studies. *Molecular Nutrition and Food Research, 56*, 159–170.
Tsuda, T., Ueno, Y., Yoshikawa, T., Kojo, H., & Osawa, T. (2006). Microarray profiling of gene expression in human adipocytes in response to anthocyanins. *Biochemical Pharmacology, 71*, 1184–1197.
Tumbas, V. T., Canadanovic-Brunet, J. M., Cetojevic-Simin, D. D., Cetkovic, G. S., Dilas, S. M., & Gillec, L. (2012). Effect of rosehip (*Rosa canina* L.) phytochemicals on stable free radicals and human cancer cells. *Journal of the Science of Food and Agriculture, 92*, 1273–1281.
Veitch, N., & Grayer, R. (2011). Flavonoids and their glycosides, including anthocyanins. *Natural Product Reports, 28*, 1626–1695.
Wang, H., Cao, G., & Prior, R. L. (1997). Oxygen radical absorbing capacity of anthocyanins. *Journal of Agricultural and Food Chemistry, 45*, 304–309.
Yoshizumi, M., Tsuchiya, K., Kirima, K., Kyaw, M., Suzaki, Y., & Tamaki, T. (2001). Quercetin inhibits Shc- and Phosphatidylinositol 3-kinase-mediated c-Jun N-terminal kinase activation by angiotensin II in cultured rat aortic smooth muscle cells. *Molecular Pharmacology, 60*, 656–665.

7.5 “Bioactivity of different enriched phenolic extracts of wild fruits from Northeastern Portugal: A comparative study”

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ORIGINAL PAPER

Bioactivity of Different Enriched Phenolic Extracts of Wild Fruits from Northeastern Portugal: A Comparative Study

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Abstract *Arbutus unedo, Prunus spinosa, Rosa micrantha and Rosa canina* are good sources of phenolic compounds, including anthocyanins. These compounds have potent antioxidant properties, which have been related to anticancer activity. Herein, the *in vitro* antioxidant and antitumor properties of enriched phenolic extracts (non-anthocyanin phenolic compounds enriched extract- PE and anthocyanins enriched extract- AE) of the mentioned wild fruits were evaluated and compared. PE gave higher bioactive properties than the corresponding AE. It was observed a high capacity of *A. unedo* phenolic extract to inhibit lipid peroxidation in animal brain homogenates (EC_{50}=7.21 μg/mL), as also a high antitumor potential against NCI-H460 human cell line (non-small lung cancer; GI_{50}=37.68 μg/mL), which could be related to the presence of galloyl derivatives (exclusively found in this species). The bioactivity of the studied wild fruits proved to be more related to the phenolic compounds profile than to the amounts present in each extract, and could be considered in the design of new formulations of dietary supplements or functional foods.

Keywords Wild fruits · Northeastern Portugal · Phenolic compounds · Anthocyanins · Antioxidant activity · Antitumor effects

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Abbreviations

DPPH	2,2-Diphenyl-1-picrylhydrazyl
FBS	Foetal bovine serum
HBSS	Hank's balanced salt solution
SRB	Sulforhodamine B
TBARS	Thiobarbituric acid reactive substances
TCA	Trichloroacetic acid

Introduction

Phenolic compounds are common constituents of fruits and vegetables that are considered an important class of antioxidant natural substances [1–3]. In fact, the interest of plant phenolic extracts derives from the evidence of their potent antioxidant activity and their wide range of pharmacologic properties including anticancer activity [4]. However, the considerable diversity of their structures affects their biological properties such as bioavailability, antioxidant activity, specific interactions with cell receptors and enzymes [5].

The antioxidant properties are conferred to phenolic compounds by hydroxyl groups attached to aromatic rings and they can act as reducing agents, hydrogen donators, singlet oxygen quenchers, superoxide radical scavengers and even as metal chelators [6]. They also activate antioxidant enzymes, reduce α-tocopherol radicals (tocopheroxyls), inhibit oxidases, mitigate nitrosative stress, and increase levels of uric acid and low molecular weight compounds [6]. For many years, phenolic compounds have been intensely studied for their antitumor, proapoptotic and antiangiogenic effects and, in recent years, the usage of these compounds has increased considerably [4]. Anthocyanins, from the flavonoids family, are found mainly in berries and have high antioxidant activity, which plays a vital role in the prevention of neuronal and cardiovascular illnesses, diabetes and cancer, among others [7].

As previously demonstrated by our research group, species such as *Arbutus unedo* L., *Prunus spinosa* L., *Rosa micrantha* Borrer ex Sm. *and Rosa canina* L. are good sources of phenolic compounds, including anthocyanins [8]. The fruits of *A. unedo* are used in folk medicine as antiseptics, diuretics and laxatives [9]. *P. spinosa* fruits have also been used as astringent, diuretic and purgative. *R. canina* fruits possess prophylactic and therapeutic activities for inflammatory disorders such as arthritis, rheumatism, gout, colds and gastrointestinal disorders [10, 11].

The antioxidant properties of extracts of *A. unedo, P. spinosa, R. micrantha* and *R. canina* fruits were previously reported by different authors [12–15], but nothing is known regarding different fractions of the mentioned extracts. Fujji et al. [16] studied the effects of an aqueous extract of *R. canina* hips on mouse melanoma cells, and demonstrated that proanthocyanidins contributed greatly to its melanogenesis-inhibiting effect on those cells. Tumbas et al. [17] reported that the flavonoids fraction from *R. canina* tea showed high antioxidant activity towards 2,2-diphenyl-1-picrylhydrazyl (DPPH), as also antiproliferative activity in three human tumor cell lines (HeLa, MCF-7 and HT-29; IC_{50} values 80.63, 248.03 and 363.95 mg/L, respectively).

Despite the mentioned studies reporting antioxidant properties of fruits of the four species, as far as we know, this is the first study regarding antitumor effects of *A. unedo*, *R. micrantha* and *P. spinosa*. Moreover, the available reports on antioxidant properties refer to crude and not purified/enriched extracts, and no conclusions could be taken about the contributions of different phenolic fractions to the bioactivity of those fruits. Therefore, in the present work, the *in vitro* antioxidant and antitumor properties of enriched phenolic extracts (non-anthocyanin phenolic compounds enriched extract and anthocyanins enriched extract) of *A. unedo, P. spinosa, R. micrantha* and *R. canina* wild fruits were evaluated and compared in order to clarify anthocyanins contribution for bioactivity and the advantageous of using purified/enriched instead of crude phenolic extracts.

Materials and Methods

Standards and Reagents

2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Foetal bovine serum (FBS), L-glutamine, Hank's balanced salt solution (HBSS), trypsin-EDTA (ethylenediaminetetraacetic acid), penicillin/streptomycin solution (100 U/mL and 100 mg/mL, respectively), RPMI-1640 and DMEM media were from Hyclone (Logan, USA). Acetic acid, ellipticine, sulforhodamine B (SRB), trypan blue, trichloroacetic acid (TCA) and Tris were from Sigma Chemical Co. (Saint Louis, USA). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

Samples

The fruits of *Arbutus unedo* L. (strawberry-tree) from Ericaceae, and the Rosaceae species *Prunus spinosa* L. (blackthorn), *Rosa canina* sl. (dog rose) and *Rosa micrantha* Borrer ex Sm. (similar to eglantine rose) were gathered in the Natural Park of Montesinho territory, in Trás-os-Montes, Northeastern Portugal. Strawberry-tree berries were collected fully ripened in November 2008; well matured blackthorn and dog rose hips were gathered in late September 2008. *R. micrantha* overripe hips, that is fleshy and soft dark red fruits, were collected in late autumn 2009. Morphological key characters from the Flora Iberica [18] were used for plant identification. The fruits with seeds were lyophilized (Ly-8-FM-ULE, Snijders, Holland) and stored in the deep-freezer at −20 °C for subsequent analysis.

Samples Preparation

Non-anthocyanin Phenolic Compounds Enriched Extract (PE) Each sample (1 g) was extracted with 30 mL of methanol:water 80:20 (*v/v*) at room temperature, 150 rpm, for 1 h. The extract was filtered through Whatman n° 4 paper. The residue was then re-extracted twice with additional 30 mL portions of methanol:water 80:20 (*v/v*). The combined extracts were evaporated at 35 °C (rotary evaporator Büchi R-210) to remove methanol. For purification, the extract solution was deposited onto a C-18 SepPak® Vac 3 cc cartridge (Phenomenex), previously activated with methanol followed by water; sugars and more polar substances were removed by passing through 10 mL of water and phenolic compounds were further eluted with 5 mL of methanol. The methanolic extract obtained (designated by phenolic extract) was concentrated under vacuum and stored at 4 °C for further use.

Anthocyanins Enriched Extract (AE) Each sample (1 g) was extracted with 30 mL of methanol containing 0.5 % TFA, and filtered through a Whatman n° 4 paper. The residue was then re-extracted twice with additional 30 mL portions of 0.5 % TFA in methanol. The combined extracts were evaporated at 35 °C to remove the methanol, and re-dissolved in water. For purification, the extract solution was deposited onto a C-18 SepPak® Vac 3 cc cartridge (Phenomenex), previously activated with methanol followed by water; sugars and more polar substances were removed by passing through 10 mL of water and anthocyanin pigments were further eluted with 5 mL of methanol:water (80:20, v/v) containing 0.1 % TFA. The methanolic extract (designated by anthocyanins extract) was concentrated under vacuum, lyophilized and stored at 4 °C for further use.

Evaluation of Bioactivity

The extracts were re-dissolved in water at a final concentration 10 mg/mL and 8 mg/mL for antioxidant and antitumor activity evaluation, respectively. The final solutions were further diluted in water to different concentrations to be submitted to distinct bioactivity evaluation *in vitro* assays (1,000–4 μg/mL and 400–25 μg/mL for antioxidant and antitumor assays, respectively). The results were expressed in *i)* EC_{50} (extract concentration providing 50 % of antioxidant activity or 0.5 of absorbance in the reducing power assay) values for antioxidant activity or *ii)* GI_{50} (extract concentration that inhibited 50 % of the net cell growth) values for antitumor activity. Water was used as negative control, and trolox and ellipticine were used as positive controls in antioxidant and antitumor activity evaluation assays, respectively.

Antioxidant Activity Assays To evaluate the antioxidant activity the following assays were used: DPPH radical-scavenging activity assay; reducing power assay; inhibition of β-carotene bleaching assay; and lipid peroxidation inhibition by thiobarbituric acid reactive substances (TBARS) assay [14, 19].

DPPH radical-scavenging activity was evaluated by using a ELX800 microplate Reader (Bio-Tek Instruments, Inc; Winooski, USA), and calculated as a percentage of DPPH discolouration using the formula: $[(A_{DPPH}-A_S)/A_{DPPH}]\times 100$, where A_S is the absorbance of the solution containing the extract at 515 nm, and A_{DPPH} is the absorbance of the DPPH solution. Reducing power was evaluated by the capacity to convert Fe^{3+} into Fe^{2+}, measuring the absorbance at 690 nm in the microplate Reader mentioned above. Inhibition of β-carotene bleaching was evaluated though the β-carotene/linoleate assay; the neutralization of linoleate free radicals avoids β-carotene bleaching, which is measured by the formula: (β-carotene absorbance after 2 h of assay/initial absorbance)×100. Lipid peroxidation inhibition in porcine (*Sus scrofa*) brain homogenates was evaluated by the decreasing in TBARS; the color intensity of the malondialdehyde-thiobarbituric acid (MDA-TBA) was measured by its absorbance at 532 nm; the inhibition ratio (%) was calculated using the following formula: $[(A-B)/A]\times 100\%$, where A and B were the absorbance of the control and the extract solution, respectively.

Antitumor Activity Five human tumor cell lines were used: MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer), HCT-15 (colon carcinoma), HeLa (cervical carcinoma) and HepG2 (hepatocellular carcinoma). Cells were routinely maintained as adherent cell cultures in RPMI-1640 medium containing 10 % heat-inactivated FBS (MCF-7, NCI-H460 and HCT-15) and 2 mM glutamine or in DMEM supplemented with 10 % FBS, 2 mM glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin (HeLa and HepG2 cells), at 37 °C, in a humidified air incubator containing 5 % CO_2. Each cell line was plated at an appropriate density (7.5×10^3 cells/well for MCF-7, NCI-H460 and HCT-15 or 1.0×10^4 cells/well for HeLa and HepG2) in 96-well plates and allowed to attach for 24 h. Cells were then treated for 48 h with various extract concentrations. Following this incubation period, the adherent cells were fixed by adding cold 10 % trichloroacetic acid (TCA, 100 μL) and incubated for 60 min at 4 °C. Plates were then washed with deionized water and dried; sulforhodamine B solution (0.1 % in 1 % acetic acid, 100 μL) was then added to each plate well and incubated for 30 min at room temperature. Unbound SRB was removed by washing with 1 % acetic acid. Plates were air-dried, the bound SRB was solubilized with 10 mM Tris (200 μL) and the absorbance was measured at 540 nm in the microplate reader mentioned above [20].

Hepatotoxicity A cell culture was prepared from a freshly harvested porcine liver obtained from a local slaughter house, and it was designed as PLP2. Briefly, the liver tissues were rinsed in Hank's balanced salt solution containing 100 U/mL penicillin, 100 μg/mL streptomycin and divided into 1×1 mm^3

Table 1 Antioxidant activity (EC_{50} values (μg/mL) corresponding to the sample concentration achieving 50 % of antioxidant activity or 0.5 of absorbance in reducing power assay) of different phenolic enriched extracts from four wild fruits (mean ± SD)

	DPPH scavenging activity	Reducing power	β-carotene bleaching inhibition	TBARS inhibition
PE				
Arbutus unedo	60.89±1.74^d	36.69±1.82^c	432.08±19.37^c	7.21±0.35^c
Prunus spinosa	64.98±6.19^c	42.08±0.66^b	641.11±80.69^b	7.39±0.20^c
Rosa canina	75.78±4.10^a	47.38±0.93^a	852.20±147.67^a	10.02±0.29^a
Rosa micrantha	69.58±3.37^b	47.80±1.19^a	755.39±82.25^b	8.89±0.26^b
AE				
Arbutus unedo	93.75±2.26^b	75.41±0.53^b	950.96±38.71^a	23.13±3.21^b
Prunus spinosa	99.37±2.36^a	83.30±0.46^a	n.a.	25.29±0.85^a
Rosa canina	81.21±2.26^d	72.75±2.38^c	893.57±29.19^b	12.39±0.18^c
Rosa micrantha	86.33±1.69^c	75.25±0.12^b	904.08±55.50^b	22.52±0.36^b
Trolox	43.03±1.71	29.62±3.15	2.63±0.14	3.73±1.90

In each column, and for each extract, different letters mean significant differences ($p<0.05$)

n.a. It was not possible to obtain EC_{50} value for this extract. *PE* Non-anthocyanin phenolic compounds enriched extract; *AE* Anthocyanins enriched extract

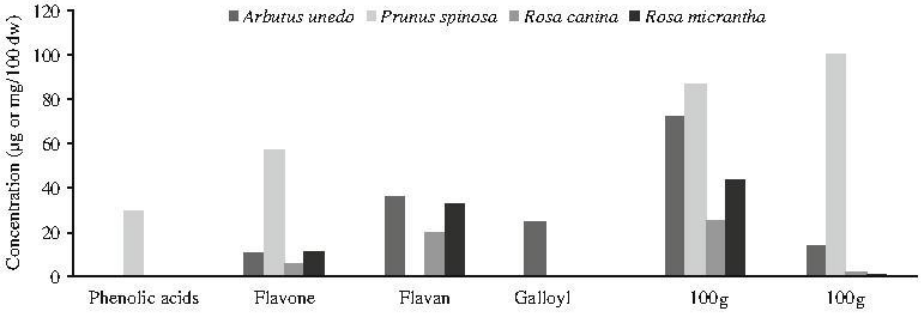

Fig. 1 Concentrations of phenolic compounds present in the wild fruits, determined by HPLC-DAD-MS/ESI according to reference [8]

explants. Some of these explants were placed in 25 cm^2 tissue flasks in DMEM medium supplemented with 10 % fetal bovine serum, 2 mM nonessential amino acids and 100 U/mL penicillin, 100 mg/mL streptomycin and incubated at 37 °C with a humidified atmosphere containing 5 % CO_2. The medium was changed every 2 days. Cultivation of the cells was continued with direct monitoring every 2 to 3 days using a phase contrast microscope. Before confluence was reached, cells were subcultured and plated in 96-well plates at a density of 1.0×10^4 cells/well, and cultivated in DMEM medium with 10 % FBS, 100 U/mL penicillin and 100 µg/mL streptomycin [20].

Statistical Analysis

All the assays were carried out in triplicate in three different extracts, and the results are expressed as mean values ± standard deviation (SD). The statistical differences represented by letters were obtained through one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference *post hoc* test with $\alpha=0.05$. These treatments were carried out using SPSS v. 18.0 program.

Results and Discussion

The results of antioxidant activity, determined by free radicals scavenging activity, reducing power and inhibition of lipid peroxidation in brain cell homogenates, are shown in Table 1. The studied extracts were chemically characterized in a previous work of our research group [8]. Herein, two different enriched phenolic extracts were prepared, in order to evaluate and compare their bioactivity: a non-anthocyanin phenolic compounds enriched extract (PE; with phenolic acids, flavones/ols, flavan-3-ols and galloyl derivatives) and a separate anthocyanins enriched extract (AE).

Regarding PE of the studied wild fruits, *A. unedo* presented the highest antioxidant activity in all the *in vitro* assays, which could be related to the presence of galloyl derivatives (exclusively in *A. unedo* PE) and/or to the presence of higher levels

Table 2 Antitumor activity and hepatotoxicity (GI_{50} values (µg/mL) corresponding to the sample concentration achieving 50 % of growth inhibition in human tumor cell lines or in liver primary culture PLP2) of different phenolic enriched extracts from four wild fruits (mean ± SD)

	MCF-7 (breast carcinoma)	NCI-H460 (non-small lung cancer)	HCT-15 (colon carcinoma)	HeLa (cervical carcinoma)	HepG2 (hepatocellular carcinoma)	PLP2 (non-tumor liver primary culture)
PE						
Arbutus unedo	153.08±10.34^d	37.68±5.02^d	93.36±5.98^c	143.36±9.07^d	128.51±7.47^d	>400
Prunus spinosa	270.65±9.25^c	154.25±6.35^c	220.44±2.89^b	193.62±11.05^c	169.56±6.39^c	>400
Rosa canina	>400^a	254.69±3.91^a	243.67±4.65^a	253.03±11.03^a	281.79±5.78^a	>400
Rosa micrantha	374.11±8.69^b	226.04±7.56^b	223.25±4.23^b	226.34±13.81^b	255.31±9.01^b	>400
AE						
Arbutus unedo	238.11±6.74	227.43±4.09^c	121.95±7.15^b	149.88±8.46^d	168.40±7.29^c	>400
Prunus spinosa	>400^a	282.92±4.28^b	234.59±6.29^a	224.58±14.09^c	231.31±9.24^b	>400
Rosa canina	>400^a	305.97±4.23^a	243.51±8.24^a	311.16±10.18^a	266.53±10.95^a	>400
Rosa micrantha	>400^a	307.68±6.05^a	264.04±3.08^a	252.07±11.01^b	270.5±9.24^a	>400
Ellipticine	0.91±0.04	1.42±0.00	1.91±0.06	1.14±0.21	3.22±0.67	2.06±0.03

In each column, and for each extract, different letters mean significant differences ($p<0.05$)

PE Non-anthocyanin phenolic compounds enriched extract; *AE* Anthocyanins enriched extract

of flavan-3-ols. The second one with the highest antioxidant effects was *P. spinosa*, in which the main contributors seemed to be phenolic acids (exclusive in *P. spinosa* PE) and flavones/ols, present in this PE in higher amounts. The studied Rosa species revealed the lowest antioxidant activity, presenting similar phenolic compounds profile (flavan-3-ols and flavones/ols); the higher levels of these compounds found in *R. micrantha* comparatively to *R. canina*, might explain the higher antioxidant activity observed in the first case (Table 1, Fig. 1).

Concerning AE, a pro-oxidant effect of anthocyanins seemed to occur, since the samples with the highest amounts revealed the lowest antioxidant activity (Table 1, Fig. 1). For that reason, *P. spinosa* gave the lowest antioxidant activity (in β-carotene-bleaching inhibition assay it was not possible to determine EC_{50} value due, in our opinion, to pro-oxidant effects of anthocyanins), while *R. canina* showed the highest antioxidant effects.

PE gave higher antioxidant properties than the corresponding AE, and according to their chemical characterization, those properties seem to be related to galloyl derivatives, flavan-3-ols, phenolic acids and flavones/ols. In general, PE and AE presented higher antioxidant activity than the methanolic extracts (crude extracts) of the same fruits previously studied by us [14, 15]. It seems that purified/enriched extracts (such as the cases herein presented) are more suitable than crude extracts, in which antagonistic effects between the compounds present could be observed, conducting to a decrease in the antioxidant activity. The only exception was for the β-carotene bleaching inhibition assay (higher capacity in crude extracts); in this case, other molecules rather than the ones previously mentioned are probably involved and might bring synergistic effects.

The antitumor potential was tested in human tumor cell lines (breast, lung, colon, cervical and hepatocellular carcinomas), and the hepatotoxicity was evaluated using a porcine liver primary cell culture. All the extracts inhibited the growth of tumor cell lines, except *R. canina* PE and AE, *P. spinosa* AE and *R. micrantha* AE for MCF-7 (breast carcinoma). *A. unedo*, followed by *P. spinosa*, PE gave the best antitumor inhibition (Table 2), which could be correlated as mentioned above for antioxidant activity (similar behaviour), to the phenolic groups present in each of the wild fruits (Fig. 1), *i.e.*, exclusive presence of galloyl derivatives and the highest levels of flavan-3-ols for *A. unedo* PE, and exclusive presence of phenolic acids and the highest levels of flavones/ols for *P. spinosa* PE. Regarding AE, samples with the highest amounts of anthocyanins (*P. spinosa* and *A. unedo*) revealed the highest antitumor effects, except in the case of MCF-7 that was not inhibited by *P. spinosa* AE. None of the samples showed toxicity for non-tumor liver primary culture.

As far as we know, this is the first study regarding antitumor effects of *A. unedo*, *R. micrantha* and *P. spinosa* wild fruits. In the case of *R. canina*, the antitumor effects of an aqueous extract from its hips were studied in mouse melanoma cells [16], and similarly to the herein studied PE, the higher contributors are proanthocyanidins (flavan-3-ols). Otherwise, the flavonoids fraction from *R. canina* tea showed higher antiproliferative activity in HeLa cell line (IC_{50}=80.63 μg/mL; [17]) than the one observed in the present study for PE (GI_{50}= 253.03 μg/mL); contrarily to the observed result (no activity up to 400 μg/mL), those authors reported effects against MCF-7 cell line (IC_{50}= 248.03 μg/mL).

Overall, the bioactivity of the studied wild fruits proved to be more related to phenolic compounds profile than to the amounts present in each extract, being PE more bioactive than AE. It should be highlighted the high capacity of *A. unedo* PE to inhibit lipid peroxidation in animal brain homogenates (EC_{50}=7.21 μg/mL), as also its antitumor potential against NCI-H460 human cell line (non-small lung cancer; GI_{50}= 37.68 μg/mL). Regarding chemical characterization of the mentioned sample, the presence of galloyl derivatives exclusively in *A. unedo* wild fruits could be related to its higher bioactivity. Further studies are needed in order to confirm the specific role of these compounds in antioxidant and antitumor effects. Due to the observed bioactive properties, the mentioned species could be considered in the design of new formulations of dietary supplements or functional foods.

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Conflict of interest The authors declare that they have no conflict of interest.

References

1. Rice-Evans CA, Miller NJ, Paganga G (1997) Antioxidant properties of phenolic compounds. Review. Trends Plant Sci 2:152–159
2. Yao LH, Jiang YM, Shi J, Tomás-Barberán FA, Datta N, Singanusong R, Chen SS (2004) Flavonoids in food and their health benefits. Plant Foods Hum Nutr 59:113–122
3. Szajdek A, Borowska EJ (2008) Bioactive compounds and health-promoting properties of berry fruits: a review. Plant Foods Hum Nutr 63:147–156
4. Carocho M, Ferreira ICFR (2013) The role of phenolic compounds in the fight against cancer- a review. Anti-Cancer Ag Med Chem 13: 1236–1258
5. Scalbert A, Williamson G (2000) Dietary intake and bioavailability of polyphenols. J Nutr 130:2073–2085
6. Carocho M, Ferreira ICFR (2013) A review on antioxidants, prooxidants and related controversy: natural and synthetic compounds, screening and analysis methodologies and future perspectives. Food Chem Toxicol 51:15–25
7. Kong JM, Chia LS, Goh NK, Chia TF, Brouillard R (2003) Analysis and biological activities of anthocyanins. Phytochemistry 64:923–933

8. Guimarães R, Barros L, Dueñas M, Carvalho AM, Queiroz MJRP, Santos- Buelga C, Ferreira ICFR (2013) Characterization of phenolic compounds in wild fruits from Northeastern Portugal. Food Chem 141:3721–3730
9. Bnouham M, Merhfour FZ, Legssyer A, Mekhfi H, Maallem S, Ziyyat A (2007) Antihyperglycemic activity of *Arbutus unedo*, *Ammoides pusilla* and *Thymelaea hirsuta*. Pharmazie 62:630–632
10. Carvalho AM (2010) *Plantas y sabiduría popular del Parque Natural de Montesinho. Un estudio etnobotánico en Portugal*. Biblioteca de Ciencias 35. Madrid: Consejo Superior de Investigaciones Científicas
11. Orhan DD, Hartevioğlu A, Küpeli E, Yesilalada E (2007) *In vivo* anti-inflammatory and antinociceptive activity of the crude extract and fractions from *Rosa canina* L. fruits. J Ethnopharmacol 112:394–400
12. Fortalezas S, Tavares L, Pimpão R, Tyagi M, Pontes V, Alves PM, McDougall G, Stewart D, Ferreira RB, Santos CN (2010) Antioxidant properties and neuroprotective capacity of strawberry tree fruits (*Arbutus unedo*). Nutrients 2:214–229
13. Mendes L, Freitas V, Baptista P, Carvalho M (2011) Comparative antihemolytic and radical scavenging activities of strawberry tree (*Arbutus unedo* L.) leaf and fruit. Food Chem Toxicol 49:2285–2291
14. Barros L, Carvalho AM, Morais JS, Ferreira ICFR (2010) Strawberry-tree, blackthorn and rose fruits: detailed characterization in nutrients and phytochemicals with antioxidant properties. Food Chem 120:247–254
15. Guimarães R, Barros L, Carvalho AM, Ferreira ICFR (2010) Studies on chemical constituents and bioactivity of *Rosa micrantha*: an alternative antioxidants source for food, pharmaceutical, or cosmetic applications. J Agric Food Chem 58:6277–6284
16. Fujii T, Ikeda K, Saito M (2011) Inhibitory effect of rose hip (*Rosa canina* L.) on melanogenesis in mouse melanoma cells and on pigmentation in brown guinea pigs. Biosci Biotechnol Biochem 75: 489–495
17. Tumbas VT, Čanadanović-Brunet JM, Četojević-Simin DD, Ćetković GS, Dilas SM, Gille L (2012) Effect of rosehip (*Rosa canina* L.) phytochemicals on stable free radicals and human cancer cells. J Sci Food Agric 92:1273–1281
18. Castroviejo S et al (2004) Flora Iberica. Plantas vasculares de la Península Ibérica e Islas Baleares, vol IV. CSIC, Real Jardín Botánico
19. Dias MI, Barros L, Sousa MJ, Ferreira ICFR (2011) Comparative study of lipophilic and hydrophilic antioxidants from *in vivo* and *in vitro* grown *Coriandrum sativum*. Plant Foods Hum Nutr 66:181–186
20. Guimarães R, Barros L, Dueñas M, Calhelha RC, Carvalho AM, Santos Buelga C, Queiroz MJRP, Ferreira ICFR (2013) Nutrients, phytochemicals and bioactivity of wild Roman chamomile: a comparison between the herb and its preparations. Food Chem 136:718–725